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LOYOLA UNIVERSITY OF CHICAGO

THE ISOLATION AND CHARACTERIZATION OF pLV22a,
A MOBILIZABLE PLASMID FROM
Bacteroides fragilis

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

MOLECULAR BIOLOGY PROGRAM

BY

THOMAS JOSEPH NOVICKI

CHICAGO, ILLINOIS

JANUARY, 1997

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF ILLUSTRATIONS	ix
LIST OF TABLES	x
Chapter	
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
Physical Characteristics of Members of the Genus <i>Bacteroides</i>	4
Epidemiology	6
Virulence Factors	9
Extracellular capsule	9
Adhesion of bacterial cells to host surfaces(cell-cell interactions)	11
Toxins	11
Miscellaneous virulence factors	12
Antibiotic Resistance Mechanisms	13
Horizontal Gene Transfer Factors	15
3. MATERIALS AND METHODS	20
Chemicals	20
Bacterial Strains	20
Bacterial Media	21
Antibiotics	22
Whole-cell DNA Preparation	23
Small-scale Plasmid DNA Preparation	24
Large-scale Plasmid DNA Preparation	25

Restriction Endonuclease Digestion	27
S1 Nuclease Digestion	27
Agarose Gel Electrophoresis	27
Extraction of DNA from Agarose Gels	28
DNA Ligation	28
Preparation and Transformation of Competent <i>E. coli</i>	29
Plasmid Constructions	31
pGAT400	31
pGAT400•BglII	34
pGAT400•ori	34
pGEM7Zf(+)	36
pBR322	36
pACYC184	36
pBR328	36
pBR328•	36
pJA-11a	38
pJA-223B	38
pJA-7ZT	38
pTJins1a	38
pTJins3a	38
pTJ5a	38
pTJ8	38
pB22a	41
pTJ6	41
pTJ6XB	41
pTJ10	41

pTJ12	41
Tn1000 Random Mutagenesis	43
Complementation Analyses	44
Quantitative <i>Bacteroides</i> to <i>E. coli</i> and <i>Bacteroides</i> to <i>Bacteroides</i> Conjugative Filter Matings	44
Quantitative R751 Mobilization	46
<i>E.coli</i> Strain Construction Using IncP Plasmid Mobilization	47
<i>Bacteroides</i> Strain Construction Using pRK231 Plasmid Mobilization	47
DNA Sequencing	48
³² P-labeled DNA Probes	49
Southern Blot Analysis	49
Polymerase Chain Reaction (PCR) Amplification of the pLV22a <i>oriT</i>	51
4. RESULTS	52
Description of the Transfer Mechanisms of the <i>B. fragilis</i> Strain LV22	52
Conjugative Transfer of the <i>Trans</i> Factor from <i>B. fragilis</i> LV22 to <i>B.</i> <i>thetaitaomicron</i> BT4001	53
Investigation of the Transfer Properties of <i>B. thetaiotaomicron</i> Strains BT22.1- BT22.50	54
Initial Analysis of the Exogenous DNA of pTJ47-1 and pTJ47-3	58
Determination of the Origin of the Exogenous DNA of pTJ47-1 and pTJ47-3	60
Investigation of the Transfer Properties of pLV22a While in <i>E. coli</i>	65
The Mobilization of pLV22a Independent of pGAT400•BglII	67

Comparison of pLV22a to the Transfer Region of pBFTM10	67
Localization of the pLV22a Mobilization Region by Tn1000 Random Insertion Mutagenesis	68
Transfer of pLV22a from <i>B. fragilis</i>	69
DNA Nucleotide Sequence of the pLV22a Mobilization Region	73
Computer Analysis of the pLV22a Mobilization Region DNA Sequence	76
Mutations in <i>orf 1</i> , <i>orf 2</i> , and <i>orf 3</i> can be Complemented in <i>trans</i>	78
Complementation analysis of mutations in <i>mbpC</i>	82
Complementation analysis of mutations in <i>mbpB</i>	83
Complementation analysis of a mutation in <i>mbpA</i>	85
Localization of a <i>cis</i> -acting Region Required for the Mobilization of pLV22a	87
Cloning the pLV22a <i>oriT</i>	89
5. SUMMARY & DISCUSSION	91
Capture of pLV22a by pGAT400-BglIII in <i>B. thetaiotaomicron</i> BT22.47	92
Characterization of the Conjugative Transfer Properties of pLV22a	95
Localization and DNA Sequence Analysis of the pLV22a Mobilization Region	100
Identification and Characterization of the pLV22a Genes Required for Mobilization in <i>E. coli</i>	102
Cloning a <i>cis</i> -acting Region Required for Mobilization	107
Potential Functions of the pLV22a Mobilization Proteins	108

WORKS CITED 114

VITA 127

LIST OF ILLUSTRATIONS

Figure	Page
1. Maps of pGAT400, pGAT400•BglIII, and pGAT400•ori	35
2. Maps of pGEM7Zf(+), pACYC184, pBR322, and pBR328	37
3. Maps of pTJins1a, pTJ5a, pTJ8, and pB22a	39
4. Maps of pTJins3a, pTJ6, pTJ6XB, and pTJ12	40
5. Map of pTJ10	42
6. <i>Ava</i> I digest patterns of <i>B. thetaiotaomicron</i> BT22 X <i>E. coli</i> HB101 transconjugant plasmids	56
7. <i>Ava</i> I digest patterns of pTJ47-1 and pTJ47-3	57
8. Map of pTJ47-1 and pTJ47-3	59
9. Southern hybridization of <i>B. fragilis</i> LV22 and <i>B. thetaiotaomicron</i> BT4001 total DNA with the 5.4 kb <i>Ava</i> I fragment of pTJ47-1	62
10. Southern hybridization of <i>B. fragilis</i> LV22, <i>B.</i> <i>thetaitotaomicron</i> BT4001 and <i>B.</i> <i>thetaitotaomicron</i> BT22.47 plasmids with the 1.8 kb <i>Sac</i> I/ <i>Eco</i> RV fragment of pTJ47-1	64
11. Map of <i>Tn1000</i> insertions in pTJ5a	70
12. DNA nucleotide and deduced protein sequences of the pLV22a mobilization region	73
13. Major open reading frames of pLV22a mobilization region	77
14. Alignment of MbpB with other known and putative relaxase protein sequences	105
15. Model of potential nucleoprotein interactions at the <i>oriT</i> of pLV22a	112

LIST OF TABLES

Table	Page
1. Bacterial strains	21
2. Plasmids	32
3. R751 mobilization of pLV22a derivatives in <i>E. coli</i>	66
4. pLV22a transfer from <i>B. fragilis</i>	72
5. R751 mobilization of Tn1000 insertion mutants in <i>E. coli</i>	80
6. Complementation analyses of pLV22a Tn1000 mutants in <i>E. coli</i>	84
7. Complementation analysis of the pLV22a <i>oriT</i> region	88

INTRODUCTION

The basis of evolutionary processes resides in the dynamic nature of genetic material: novel DNA sequences are continually being produced by mutation and recombination. Through the stabilization and transmission of these genetic products, evolutionary directions are established. Vertical transmission, the transfer of genes from parents to offspring, is most generally thought to determine these directions. However, it is becoming increasingly apparent that another form of transmission, *horizontal transfer*, contributes greatly to evolutionary processes in a wide variety of organisms.

Horizontal gene transfer refers to the mobilization and stabilization of genetic information from one organism to another. Such exchange is a well-established phenomenon in the prokaryotic kingdom. Among bacteria, genetically plastic transmissible elements are mobilized into host organisms, where modifications and further radial transfer can occur. Mobilization primarily involves extrachromosomal elements, many of which encode systems for their own transfer. (*Emphasis added.*) (From Bacterial Plasmids and Gene Flux (2).)

This dissertation project has sought to elucidate the mechanism of horizontal gene transfer of pLV22a, a 4.2 kb plasmid isolated from *Bacteroides fragilis* strain LV22. The project is comprised of 5 main areas of investigation. In the first part of the project, pLV22a was captured by the *E. coli*-*Bacteroides* shuttle plasmid pGAT400-BglIII in a series of experiments designed to investigate multiple transfer factors native to strain LV22. Fusion of these 2

plasmids resulted in a conjugatively transferable phenotype (i.e., transferable by a conjugation-type mechanism). The second line of investigation demonstrated that: (a) pLV22a can transfer independently in *B. fragilis*; (b) pLV22a can transfer in *Escherichia coli*, when co-resident with the broad-host-range conjugal (i.e., self-transferable) IncP β plasmid R751.

The third line of investigation sought to define the region required for pLV22a transfer in *E. coli* through the use of Tn1000 transposon mutagenesis. A 1.5 kb region was identified, and its nucleotide sequence determined.

The fourth area of investigation identified, using computer analysis, five open reading frames of greater than 10 kDa; 3 in the 'top' strand, and 2 in the 'bottom' strand. Complementation analysis was then applied to determine which of the 3 top-strand open reading frames were required for transfer in *E. coli*. All 3 open reading frames were found to be independently expressed and necessary for transfer.

The fifth line of research utilized sequence and complementation analyses to localize the *cis*-acting pLV22a origin of transfer (*oriT*) to a region within the 1.5 kb transfer region. (The *oriT* is the point at which one strand of DNA is specifically nicked in preparation for transfer.) Using the polymerase chain reaction and complementation analysis, the *oriT* region was cloned, and

subsequently found to confer transferability to a transfer-deficient *E. coli* vector.

CHAPTER 2

LITERATURE REVIEW

Physical Characteristics of Members of the Genus *Bacteroides*

Bacteroides are obligate anaerobic gram negative rod bacteria (98). *Bacteroides* require anaerobic conditions for growth, but are also highly aerotolerant, and can survive prolonged exposure to oxygen. They are found in high concentrations in the colon of both humans and animals. Members of the genus include *B. fragilis*, *Bacteroides caccae*, *Bacteroides distasonis*, *Bacteroides eggerthii*, *Bacteroides merdae*, *Bacteroides ovatus*, *Bacteroides stercoris*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis* and *Bacteroides vulgatus* (97).

Bacteroides are highly saccharolytic organisms. In the gut, most mono- and disaccharides are efficiently absorbed in the small intestine, and do not reach the colon, where *Bacteroides* are found (85). Nevertheless, these organisms are able to flourish by hydrolyzing a variety of complex polysaccharides from both host-ingested plant material and host secretions (87). Some of the enzymes involved in polysaccharide degradation include

neuraminidase, α -glucosidase, β -glucosidase, α -fucosidase, α -N-acetylgalactosaminidase, β -N-acetylglucosaminidase, α -galactosidase, β -galactosidase, chondroitin lyase I, and chondroitin lyase II (6,33,38,52). The majority of these enzymes have been found to be associated with the outer membrane and periplasm, while others are cytosolic in nature. In the laboratory, most *Bacteroides* grow readily on a simple defined medium which consists of a fermentable carbohydrate, a source of nitrogen such as ammonium sulfate, vitamin B₁₂, hemin, inorganic ions and cysteine (as a reductant). Growth rates are about 0.33-1 generation per hour (85). The pathways used by *Bacteroides* in the metabolism of absorbed carbohydrates have not been fully defined, but include Embden-Meyerhof glucose fermentation, (53), a glucose \rightarrow succinate metabolic pathway (98), and possibly a pentose phosphate-hexose monophosphate shunt pathway (99).

Bacteroides cannot use amino acids or proteins as the sole source of carbon or energy, nor do they require amino acids or peptides when grown on carbohydrates (85). Nevertheless, several *Bacteroides* extracellular peptidases have been identified. Gibson et al. have characterized 3 major proteases in a strain of *B. fragilis*. P1 is a serine protease, and is located intracellularly and in the periplasm. P2, a metalloprotease, and P3, a cysteine

protease, are located in the outer membrane (32). The activity of these proteases was found to progressively transfer into the culture medium during stationary phase. Whether this transfer represented active enzyme secretion or simple cell degradation was not determined (31). In a study of proteolytic gut bacteria, they found *Bacteroides* to be well represented (51).

Since protein catabolism is not required for growth, the protease production found in *Bacteroides* is intriguing. The ability to use both carbohydrates and peptides for protein and lipid synthesis may give an advantage to these organisms in the colon, where host dietary intake is in constant flux. For example, Smith and Salyers have found that *B. thetaiotaomicron* can ingest amino acids and incorporate them *de novo* into proteins and lipids (110). *Bacteroides* proteases may also be involved in the pathogenesis of these organisms, as discussed in Miscellaneous Virulence Factors (Chapter 2).

Epidemiology

Bacteroides are the predominant anaerobes in the human gut (93), and account for approximately 21% of all colonic isolates (58). As such, they are considered to be important contributors to the maintenance of the colonic ecosystem. *Bacteroides* produce fermentation products that serve as nutrients for other colonic microflora as well as

the host (85), and may also contribute to colonic dietary fiber digestion (86).

While *Bacteroides* play a prominent role in the colonic ecosystem of their host, they frequently become opportunistic pathogens when released into the surrounding tissues. They are the predominant anaerobic isolates from abdominal infections, skin and subcutaneous wounds, and abscesses proximal to the rectum (13). The reason for the frequent appearance of *Bacteroides* in these infections may be due in part to their high concentration in the colon. However, virulence factors also play a significant role in their pathogenicity (see Virulence Factors, Chapter 2). A retrospective study by Brook on the distribution of different *Bacteroides* species in clinical isolates over a 12 year period at Walter Reed Army Medical Center in Washington, DC and the Navy Hospital in Bethesda, MD drew several conclusions (12). Among them, out of 15844 bacterial cultures collected from patients at these 2 hospitals in the 12 year period, *Bacteroides* were isolated from 19% of them. *B. fragilis* accounted for 63% of all *Bacteroides* isolates; *B. thetaiotaomicron* for 14%; *B. vulgatus* and *B. ovatus* for 7% each; *B. distasonis* for 6%; and *B. uniformis* for 2%. Of the five major foci for *Bacteroides* infection, *B. fragilis* was isolated with the greatest frequency: 78% of all *Bacteroides* species from blood cultures, 69% from wounds, 65% from abscesses, 59%

from abdominal infections, and 56% from pelvic infections. Members of the genus were also regularly isolated from chest and genitourinary tract infections, cysts, bile, and tumors. *Bacteroides* septicemia (i.e., persistent invasion of the blood stream by bacteria) frequently had an unfavorable outcome, with an overall mortality of 24% (14).

Infections involving *Bacteroides* are often polymicrobial in nature. In addition to *Bacteroides*, rectal abscesses also regularly contain *Staphylococcus aureus*, *Streptococcus pyogenes*, or Enterobacteriaceae (especially *E. coli*). Likewise, abdominal abscesses containing *Bacteroides* frequently also contain *E. coli* or other Enterobacteriaceae (13). An exception to this is in *Bacteroides* septicemia, where infections can be poly- or monomicrobial (14,19). Although the organisms co-isolated with *Bacteroides* are usually a part of the normal microflora surrounding the infection foci, their appearance along with *Bacteroides* is probably not coincidental. Synergy between *Bacteroides* and *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, *S. pyogenes*, and enterococci has been noted. *In vivo* studies using murine models have found that this synergy can lead to a lowered Lethal Dose₅₀ (16), an enhanced ability to form abscesses (15,16), an induction of at least 1 *Bacteroides* virulence factor (i.e., capsule

formation) (15) and an increase in the survival of organisms in abscesses (11).

Bacteroides possess a wide array of tools with which they cause and maintain infections. These include virulence factors, antibiotic resistance mechanisms, and horizontal gene transfer factors.

Virulence Factors

A virulence factor is defined as a bacterial attribute or product that provides the organism with a selective advantage for establishing infection or ensuring its own reproduction (44). Known or putative bacterial virulence factors include extracellular capsules, fimbriae, outer membrane proteins, lipopolysaccharides, proteases, nucleases and other enzymes, metabolites and exotoxins. *Bacteroides* possess both confirmed and putative virulence factors.

Extracellular Capsule. Perhaps the best-studied *Bacteroides* virulence factor is their extracellular mucopolysaccharide capsule, common to all *Bacteroides* species with the possible exception of *B. distasonis* (3,15). The capsule is not a homogenous entity, showing variation at the species and strain levels. Pantosti et al. have identified 2 antigenically distinct capsular polysaccharides in a single *B. fragilis* strain,

polysaccharide A and polysaccharide B, while Patrick et al. have classified large-capsule, small-capsule and electron-dense capsule populations in different *B. fragilis* strains (50,72,73,116). *B. thetaiotaomicron* and *B. vulgatus* isolates possessing unique capsule configurations have also been identified. These isolates displayed numerous strands of polysaccharide extending from the cell surface, and were highly resistant to host-mediated killing (7).

Capsule formation is induced by unknown signals present *in vivo*: animal passage leads to increased encapsulation, as does co-infection with aerobic bacteria. Conversely, serial subculture reduces capsular expression (15,108). *In vivo*, the capsule appears to enhance infectivity, since intraperitoneal injections of encapsulated *B. fragilis* strains alone produce abscesses in rats, while unencapsulated strains usually require co-infection with aerobic bacteria (64). The capsule itself is a causative agent, since capsule material alone can stimulate intra-abdominal abscess formation in rats (64).

Capsules also influence the host-mediated response to *Bacteroides* infection. Unencapsulated strains have increased susceptibility to host-mediated opsonophagocytic killing (80,108), and *Bacteroides* capsular preparations alone can inhibit polymorphonuclear leukocyte metabolism, chemotaxis (27), and phagocytosis (119). Capsule

preparations also confer immunity to subsequent *Bacteroides* challenge (100).

Adhesion of bacterial cells to host surfaces (cell-cell interactions). Adhesion of gram-negative bacteria to mucosal surfaces, resulting in enhanced bacterial retention and thus infectivity, is generally associated with fimbriae (44). Pruzzo et al. found that *B. fragilis* fimbriae increased non-specific bacterial cell adherence to both epithelial cells and neutrophils, which in turn led to increased phagocytosis by the latter (75,76). The net contribution of these structures upon the virulence of *B. fragilis* is consequently unclear. Oyston and Handley reported that the capsule itself may play a significant role in *Bacteroides* adhesion (66), while Guzman et al. have established that 48% of *B. fragilis* clinical isolates studied possessed a neuraminidase-dependent adhesin that promoted attachment to mammalian epithelial cells, but not human polymorphonuclear leukocytes (40). Since *B. fragilis* produces neuraminidase, this adhesin may be physiologically important for cell-cell attachment (33,52).

Toxins. While *B. fragilis* contains lipopolysaccharide (LPS) in its cell wall, the LPS has a low endotoxic activity when compared with enterobacterial

LPS (121). While not toxic, Zaleznik et al. found that *B. fragilis* LPS is a potent abscess inducer, and thus can be classified as a virulence factor (125).

Bacteroides do produce exotoxins. Weikel et al. reported that *B. fragilis* isolates from patients exhibiting diarrhea were enterotoxigenic (120). In separate studies, Pantosti et al. and Van Tassell et al. demonstrated that human and animal clinical isolates of *B. fragilis* frequently produced exotoxins that were both entero- and cytotoxic (71,118).

Miscellaneous virulence factors. Other factors which may contribute to *Bacteroides* virulence include: (a) succinic acid, a metabolic by-product of *Bacteroides* glucose catabolism. *In vitro*, succinic acid inhibited polymorphonuclear leukocytes in a system that mimicked conditions found in clinical abscesses. Killing of *E. coli* was eliminated, and both random migration and directed chemotaxis of leukocytes was impaired (82,83). (b) Farias et al. reported a strain of *B. fragilis* that produced 2 bacteriocidal proteins. These proteins, termed fragilisins, could give strains producing them a competitive advantage during pathogenesis (26). (c) McGregor et al. showed that many clinical isolates of *B. fragilis* and *B. thetaiotaomicron* produced phospholipase C. This enzyme hydrolyzes lecithin, a cell membrane

component, and may contribute to host tissue damage and enhance bacterial invasiveness (55). (d) The production of proteases have already been discussed in conjunction with *Bacteroides* metabolism. *Bacteroides* do not require amino acids for growth, yet produce many proteases.

Proteases are likely virulence factor candidates, as they have the potential to degrade a variety of host proteins important in defense and cell wall assembly. In one case, a metalloprotease has been found to be enterotoxigenic (57).

(e) Iron-repressible outer membrane proteins

(IROMPs). Iron is necessary for bacterial growth, and must be collected from the extracellular environment.

IROMPs are present in the outer membranes of *B. fragilis* grown in iron-poor media (44,65), and may give *Bacteroides* a competitive advantage over other bacteria in the iron-poor milieu of the colon.

Antibiotic Resistance Mechanisms

The aforementioned virulence factors act together coordinately to increase the pathogenicity of *Bacteroides* throughout the course of an infection. In contrast, antibiotic resistance, while adding to the overall virulence of an organism, typically does not come into play until an infection has been established and treatment initiated. (An exception to this may be the intermittent exposure to "naturally" produced antibiotics, such as

penicillin and streptomycin.) Thus, while being intrinsically different from other virulence factors, antibiotic resistance does influence the final outcome of an infection (46).

Bacteroides are universally resistant to aminoglycosides (17). Most strains are also resistant to tetracycline, and most β -lactam drugs including the penicillins and many cephalosporins (13,86). In addition, Garcia-Rodriguez et al. found varying levels of resistance to cefoxitin (2-63 %), clindamycin (0-34%), and piperacillin (12-21 %) in a recent, retrospective world-wide survey. Chloramphenicol, while no longer generally used to treat *Bacteroides* infections due to its potential for toxicity, was nevertheless found active against most clinical isolates (30). Imipenem and metronidazole, along with β lactam/ β lactamase inhibitor combinations (e.g., amoxicillin/clavulanic acid, ampicillin/sulbactam, ticarcillin/clavulanate and piperacillin/tazobactam) are thus considered the best therapeutic choices for the treatment of *Bacteroides* infections.

The antibiotic resistance mechanisms found in *Bacteroides* vary widely according to the class of drug under consideration. Aminoglycoside resistance is due to a lack of drug transport into the cell (17). Resistance to penicillins/cephalosporins is primarily mediated through the production of a variety of β -lactamases

(47,63,114), while ribosomal protection mechanisms account for clindamycin (78) and tetracycline (61) resistance.

Horizontal Gene Transfer Factors

Many bacterial plasmids, and occasionally chromosomal factors, are transferable to adjacent cells by a mating process collectively known as conjugation. These transfer factors often carry antibiotic resistance genes, and thus can account for the rapid conversion of an antibiotic-sensitive bacterial population to a resistant one.

Examples of *Bacteroides* transferable resistance factors are: pBFTM10 (15 kb) (113), pBF4 (41 kb) (105), and pBI136 (82 kb) (109), all of which bear clindamycin resistance genes on related mobile DNA elements; pRYC3373 (40 kb) which carries a chloramphenicol resistance gene (54); the family of chromosomal factors known as Tet Elements (70-200 kb), which carry tetracycline resistance genes, with or without closely linked erythromycin resistance genes (4,102). *B. fragilis* strain TAL4170 (22), and *B. thetaiotaomicron* UN101 (77), also appear to possess chromosomal cefoxitin resistance transfer factors.

Some conjugative (i.e., transferable by a conjugation-type mechanism) factors appear to carry no adventitious genes, and are termed cryptic. While the functions of cryptic transfer factors in *Bacteroides* are unknown, their widespread occurrence suggests they do play

a role in horizontal gene transfer. Cryptic transfer factors may carry genes that confer a growth advantage only in specific environments, and have the potential to act in conjunction with mobile DNA elements to acquire and then transfer new adventitious genes. Among the characterized *Bacteroides* cryptic transfer factors are XBU4422, a 65 kb chromosomal element related to the Tet Elements (107), the plasmids pB8-51 (4 kb) (104,117), and pBI143 (3 kb) (48), and the chromosomal insertion elements NBU1 and NBU2 (10-12 kb) (48,106). A cryptic transposon, Tn4399 (10 kb), has also been described (42,43,59).

Conjugative transfer of DNA is a multi-step process that requires many functions including mating pair formation and maintenance, and DNA processing in both the host and recipient cells (28,124). In the case of large transferable plasmids such as F and R751 from the family Enterobacteriaceae, most of these functions are supplied by the plasmid, and they are referred to as conjugal or self-transferable plasmids. In contrast, small plasmids like ColE1 from *E. coli* perform only minimal DNA processing, while other required functions are provided by a co-resident conjugal plasmid. These small plasmids are referred to as mobilizable plasmids, and cannot transfer unassisted. Mobilization may thus be seen as a process in which the mobilized plasmid fortuitously uses the conjugation system of the co-resident conjugal plasmid.

Both conjugal and mobilizable plasmids that have been characterized possess 2 components absolutely required for transfer: a *cis*-acting region of DNA known as the origin of transfer (*oriT*), and 1 or more *trans*-acting DNA processing proteins, including a DNA relaxase. Relaxases bind at their cognate *oriT*, and then specifically cleave 1 strand of DNA at a nick site (*nic*) within *oriT*. After nicking, the strand is transferred to the recipient cell (8,25,28,29,45,49,115,124). This protein-DNA recognition has invariably been shown to be unique for a given plasmid class (i.e., the nicking proteins of 1 plasmid type will not recognize the *oriT* of other plasmid types).

Possibly the best characterized DNA processing model system is that of RP4, a conjugal IncP α broad-host-range plasmid. The generation of a single DNA strand destined to be transferred to a recipient cell begins when complex nucleoprotein structures called relaxosomes form in a defined sequence at the *oriT* of RP4 (67). First, TraJ recognizes, and then binds, to the right half of a 19 bp inverted repeat sequence within the *oriT* (126). Then, TraI recognizes a 6 bp sequence between *nic* and the TraJ-binding site, and joins the TraJ-*oriT* nucleoprotein complex (69). TraH specifically interacts with both TraI and TraJ, and is proposed to stabilize the relaxosome (67). Another protein, TraK, acts to enhance nicking at *oriT* (29). Several molecules of TraK have been found to

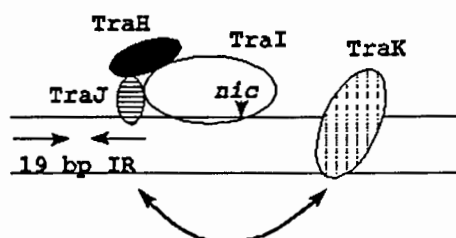
bind to a region of bent DNA adjacent to the *nic* site, resulting in a local alteration of DNA superhelicity (127). Once the relaxosome is formed, TraI cleaves a defined strand of

DNA at *nic*, after which TraI becomes covalently attached to the 5' phosphoryl end

(69). TraI is also thought to act in transfer termination in an undefined way

(70). This sequence of events leading to single-strand DNA transfer is not unique to RP4. Similar DNA-protein interactions at *oriT* have been found in other plasmid transfer systems as well (8,36,45,49,95).

The sequence of events leading up to the transfer of DNA has not been fully elucidated in any *Bacteroides* plasmid to date. However, the RP4 relaxosome model may be applicable to at least some *Bacteroides* factors. The transfer regions of 3 *Bacteroides* plasmids have been partially characterized, and all possess some components analogous to those of the RP4 system. In the first example, Hecht et al. have identified 2 genes in the *B.*



Schema for proposed nucleo-
protein interactions at the
RP4 *oriT*

fragilis clindamycin resistance plasmid pBFTM10, *btgA* and *btgB*, that are required for mobilization in *E. coli* when co-resident with R751, and for self-transfer in *B. fragilis* (41). Both of these genes, and a *cis*-acting *oriT* as well have been cloned. *BtgA*, the protein product of *btgA*, has been found to specifically bind *oriT*_{pBFTM10}, and is thought to play a part in the initiation of transfer (D.W. Hecht, personal communication). In the second example, Murphy and Malamy have characterized the mobilization region of the *B. fragilis* conjugative transposon Tn4399 (59). They have identified 2 genes required for mobilization, *mocA* and *mocB*, and an *oriT* (59). *MocA*, and *MocB* are both necessary for site- and strand-specific nicking at *oriT*_{Tn4399} (60). Finally, Li et al. have characterized the mobilization region of the *Bacteroides* chromosomal insertion element NBU 1 (48). They have identified an *oriT*, and a gene, *mob*, required for mobilization in *Bacteroides* when co-resident with a conjugal Tet Element. In this case, the protein product of *mob* bears no homology to known relaxases, but may participate in DNA nicking at *oriT*.

CHAPTER 3

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma Chemicals or J.T. Baker unless otherwise noted.

Bacterial Strains

The bacterial strains and their genotypes are given in table 1.

B. thetaiotaomicron BT4001 is a spontaneous rifampicin-resistant mutant of *B. thetaiotaomicron* type strain 5482 and was generously provided by Abigail Salyers of the University of Illinois, Urbana. *B. fragilis* TM4000 is also known as *B. fragilis* type strain 638rfm. *B. fragilis* TM4.23 is a variant of *B. fragilis* TM4000 that contains the TM230 chromosomal Tet element. TM4000 and TM4.23 were generously provided by Michael Malamy of Tufts University. *B. fragilis* LV22 was isolated at a hospital in Las Vegas, Nevada, and is part of a collection in the D.W. Hecht laboratory.

Table 1.--Bacterial strains

Strain	Relevant phenotypes ^a	Source
<u><i>Bacteroides</i> spp.</u>		
<i>B. fragilis</i> LV22	Tc ^r	This study
<i>B. fragilis</i> TM4000	Rif ^r	M. Sebald, Pasteur Institute, Paris France, as 638rfm
<i>B. fragilis</i> TM4.23	Tc ^r , Rif ^r , <i>B. fragilis</i> TM4000 + TMP230 Tet Element	Hecht, D.W. & Malamy, M. (42)
<i>B. thetaiotaomicron</i> BT4001	Rif ^r	Shoemaker, N. & Salyers, A. (106)
<i>B. thetaiotaomicron</i> BT22.47	Tc ^r , Rif ^r	This study
<u><i>Escherichia coli</i></u>		
<i>E. coli</i> HB101	Sm ^r , <i>recA</i> 13	Sambrook et al. (90)
<i>E. coli</i> DH5 α	Nal ^r , <i>recA</i> 1	Gibco BRL, Gaithersburg, MD USA
<i>E. coli</i> DW1030	Sp ^r , <i>recA</i> 13	Robillard et al. (81)

Note:^a Nal^r, Rif^r, Sp^r, Sm^r, and Tc^r indicate resistance to nalidixic acid, rifampicin, spectinomycin, streptomycin and tetracycline, respectively.

Bacterial Media

Bacteroides were grown in BHIS medium, (3.7% (w/v) BHI medium (BBL), 0.5% (w/v) yeast extract (Difco), 0.0005% (w/v) hemin) at 37° C under anaerobic conditions (5% CO₂, 10% H₂, 85% N₃). *E. coli* was grown in modified Luria (ML) medium (1.0% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, 0.2% (w/v) K₂HPO₄)

at 37° C. Agar (Difco) was added to BHIS and ML broths at 1.5% (w/v) for plating.

Antibiotics

Antibiotic concentrations used for selection of strains and plasmids were: ampicillin (Marsam Pharmaceuticals), 200 µg/ml; chloramphenicol, 40 µg/ml; clindamycin (Upjohn), 12 µg/ml; gentamicin (Elkins-Sinn), 40 µg/ml; kanamycin, 50 µg/ml (25 µg/ml when used in combination with other antibiotics); streptomycin, 50 µg/ml; spectinomycin, 50 µg/ml; and tetracycline, 10 µg/ml (*E. coli*) or 5 µg/ml (*Bacteroides*). *E. coli* strains containing R751 were grown on Mueller-Hinton medium supplemented with 10 µg/ml trimethoprim. Ampicillin, clindamycin, kanamycin, streptomycin, and spectinomycin were dissolved in water, filter-sterilized, and stored at -20° C. Gentamicin was supplied in a sterile, aqueous solution and stored at room temperature. Tetracycline was dissolved in 95% (v/v) EtOH (Aldrich) and stored at -20° C. Trimethoprim was dissolved in 70% (v/v) EtOH (Aldrich) and chloramphenicol was dissolved in 95% (v/v) EtOH (Aldrich). Both were stored at 4° C. Aqueous antibiotic solutions were used within 1 year of preparation; ethanolic solutions were used within 2 weeks.

Whole-cell DNA Preparation

Whole-cell DNA was prepared essentially as described by Saito and Muira (84). A 10 ml volume of saturated *Bacteroides* or *E. coli* culture was transferred to a 40 ml Oakridge tube, and collected by centrifugation (7.8 kRCF_{ave}, 10 min, at 4° C). The cells were washed once in 50 ml of saline-EDTA (0.15 M NaCl, 0.1 M EDTA pH 8.0), collected by centrifugation, and suspended in 5 ml of saline-EDTA supplemented with 4 mg of lysozyme. The cells were incubated 20 min at 37° C and then quick-frozen in an EtOH/dry ice bath. 5 ml of Tris-SDS buffer (0.1 M Tris pH 9.0, 1% sodium dodecyl sulfate, 0.1 M NaCl) was added, and the cells thawed with gentle mixing in a 50° C water bath. The lysate was then diluted with 5 ml of saline-EDTA. 5 ml of phenol (United States Biochemical):chloroform:isoamyl alcohol (25:24:1) was added to the lysate, the mixture briefly shaken and placed on ice for 15 min. The lysate was then centrifuged at 11 kRCF_{ave} for 30 min at 20° C. The aqueous phase was transferred to a fresh Oakridge tube. The phenol extraction step was repeated once if *Bacteroides* cells were used. 1 volume of 2-propanol was added and the mixture incubated 30 min at R.T. The precipitated nucleic acids were then collected by centrifugation (15.6 kRCF_{ave}, 15 min, 20° C), the supernate removed, and the nucleic acids dried in a vacuum desiccator for 30 min at room temperature (R.T.). They

were then dissolved in 1 ml TE (30 mM Tris pH 8.0, 5 mM EDTA) supplemented with 40 µg/ml of DNase-free RNase A, and incubated 30 min at 37° C to digest RNA.

Small-scale Plasmid DNA Preparation

Plasmid DNA for endonuclease restriction analysis as well as some DNA sequencing was prepared by miniprep alkaline lysis as described by Sambrook et al. (88). In a microcentrifuge tube, 1.5 ml of saturated *E. coli* culture or 3.0 ml of saturated *Bacteroides* culture was briefly spun in a microcentrifuge. The supernate was then removed, and the cells suspended in 100 µl of Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0). To lyse the cells, 150 µl of fresh Solution 2 (0.2 M NaOH, 1% (w/v) sodium dodecyl sulfate) was added, mixed gently, and placed on ice for 5 min. 150 µl of Solution 3 (5 M potassium acetate, pH 4.8) was added, mixed gently, and the mixture again iced for 5 min. The mixture was then centrifuged for 15 min at 4° C. The supernate, containing plasmid DNA, cellular RNA, and protein, was transferred to a new microcentrifuge tube and extracted once with phenol (United States Biochemical):chloroform (1:1). If a *Bacteroides* strain was lysed, the phenol extraction was repeated once. The aqueous phase was then transferred to a new microcentrifuge tube, and 2 volumes of 95% (v/v) EtOH (Aldrich) added. The solution was kept

at -70°C for 5 min, and the nucleic acids collected by centrifugation in a microcentrifuge. The supernate was drained, the nucleic acid pellet washed with ice-cold 70% (v/v) EtOH (Aldrich), and the pellet dried for 5 min at 37°C . The nucleic acids were then dissolved in TE (30 mM Tris pH 8.0, 5 mM EDTA) supplemented with 40 $\mu\text{g/ml}$ of DNase-free RNase A, and the solution incubated for 30 min at 37°C to digest RNA species.

When preparing mini-prep plasmid DNA for sequencing, the following modifications were made: (a) 5 ml of bacterial culture was used instead of 1.5 ml; (b) cleared lysates were extracted twice with phenol:chloroform, followed by an extraction with chloroform.

Large-scale Plasmid DNA Preparation

Large-scale plasmid DNA was prepared by 2 methods: (a) Qiagen affinity column purification (Qiagen Corp), as instructed by the manufacturer; (b) large-scale alkaline lysis with CsCl equilibrium gradient purification essentially as described by Sambrook et al. (90). The latter procedure was performed as follows: a 100 ml culture of *E. coli*, containing the plasmid to be purified, was grown to saturation with antibiotic added for plasmid maintenance. The cells were collected by centrifugation (10.2 kRCF_{ave}, 10 min, 4°C), suspended in 10 ml of Solution 1 (50 mM glucose, 25 mM Tris pH 8.0, 5 mg/ml

lysozyme, 10 mM EDTA), transferred to a 40 ml Oakridge tube, and incubated for 5 min at R.T. To lyse the cells, 20 ml of fresh Solution 2 (0.2 M NaOH, 1% (w/v) sodium dodecyl sulfate) was added and mixed gently, and the solution incubated at R.T. for 10 min. 15 ml of Solution 3 (5 M potassium acetate, pH 4.8) was added, mixed, and the solution placed on ice for 10 min. The mixture was then centrifuged for 30 min at 26.7 kRCF_{ave} , 4° C . The supernate was transferred into two 40 ml Oakridge tubes, and 0.6 volumes of 2-propanol added to each tube to precipitate nucleic acids. The tubes were incubated for 15 min at R.T. and centrifuged for 30 min at 26.7 kRCF_{ave} , 20° C . The supernates were then drained, and the nucleic acids dissolved in 7.4 ml TE (30 mM Tris pH 8.0, 5 mM EDTA). To isolate and purify the plasmid DNA, CsCl equilibrium gradient separation was performed. To 7.4 ml of nucleic acid solution, 1 g of CsCl and 0.8 ml of 10 mg/ml ethidium bromide were added, mixed, and centrifuged for 16 h at 183.2 kRCF_{max} , 20° C . The plasmid bands were then harvested from the CsCl gradients by side puncture, pooled, and the ethidium bromide extracted with 2-propanol saturated with 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate pH 7.0). The DNA was then precipitated in a 40 ml Oakridge tube by adding an equal volume of TE and 2 volumes of 95% (v/v) EtOH (Aldrich) and overnight storage at -20° C . The precipitated DNA was then collected by

centrifugation (15.6 kRCF_{ave}, 30 min, at 4° C), the supernate removed, the pellet dried in a vacuum desiccator, and then suspended in 2 ml TE.

Restriction Endonuclease Digestion

Restriction endonuclease digestions were performed as described in Sambrook et al. (89), using enzymes and reaction buffers supplied by New England Biolabs, Promega, or Stratagene.

S1 Nuclease Digestion

In some instances, S1 nuclease (Promega) was used to generate blunt-ended DNA prior to ligation. Digestions were typically done in a 50 µl volume in S1 nuclease buffer (0.25 M NaCl, 15 mM acetic acid, 15 mM potassium acetate pH 4.5, 1 mM ZnSO₄, 5% (v/v) glycerol) using 25 units of enzyme. The mixture was incubated for 1 h at R.T. and the DNA purified by agarose gel electrophoresis.

Agarose Gel Electrophoresis

Gel electrophoresis was performed using agarose concentrations of 0.6%-2.0% according to DNA fragment size, and 1X SB3 running buffer (37.5 mM Tris, 8.0 mM sodium acetate, 0.8 mM EDTA, pH 7.9). Gel slabs were then stained with 0.5 µg/ml of ethidium bromide and the DNA visualized by short-wave UV light.

Extraction of DNA from Agarose Gels

DNA fragments that were to be used in molecular cloning procedures were generally purified by agarose gel electrophoresis and then extracted from the agarose with the Gene-Clean II System (Bio 101) if greater than 500 bp in size, or with the Wizard PCR Prep Purification System (Promega) if less than 500 bp. Both systems were used as instructed by their manufacturers.

DNA Ligation

Cohesive-end ligation of DNA fragments was performed as described by Sambrook et al. (91), using 1.5 U of T4 DNA ligase and buffer supplied by New England Biolabs or Promega, for 4 hr or overnight at 16° C.

Blunt-end ligations were performed as described by Sambrook et al. (91), using T4 DNA ligase, supplied by New England Biolabs or Promega, and blunt-end ligation buffer (33 mM Tris pH 7.6, 3 mM MgCl₂, 5% (w/v) PEG8000, 0.1 mM ATP, and 5 mM DTT), overnight at R.T.

Prior to ligation, the vector DNA was usually de-phosphorylated at its 5' ends using calf intestinal alkaline phosphatase as recommended by the supplier (New England Biolabs). Ligated DNA was routinely transformed to competent *E. coli* DH5 α .

Preparation and Transformation of Competent *E. coli*

E. coli strains were made competent prior to DNA transformation using two protocols. The *E. coli* strains DH5 α and HB101 were used on a regular basis for the transformation of DNA. Consequently, competent cell preparations of these 2 strains were prepared and stored frozen, using the following method: from a bacterial culture grown to saturation, 1 ml of cells were sub-cultured to 100 ml of pre-warmed Y broth (0.5% (w/v) yeast extract (Difco), 2% (w/v) tryptone (Difco) and 0.5% (w/v) MgSO₄, adjusted to pH 7.6 with KOH) and incubated at 37° C with vigorous shaking, until the density of cells reached an OD₅₅₀ of 0.48. The cells were then chilled on ice for 5 min, and collected by centrifugation (3.7 kRCF_{ave}, 10 min, 4° C). The supernate was then drained, the cells suspended in 40 ml of ice-cold TBF1 solution (30 mM potassium acetate, 100 mM KCl, 10 mM CaCl₂, 50 mM MnCl₂, and 15% (v/v) glycerol, adjusted to pH 5.8 with acetic acid) transferred to a 40 ml Oakridge tube, and chilled on ice for 5 min. The cells were collected by centrifugation (7.8 kRCF_{ave}, 10 min, 4° C), the supernate removed, and the cells suspended in 4 ml of TBF2 solution (10 mM MOPS, 75 mM CaCl₂, 10 mM KCl, and 15% (v/v) glycerol, adjusted to pH 6.5 with KOH) and chilled on ice for 15 min. The cells were then frozen at -70° C. To transform, frozen cells were thawed and placed on ice for 10 min. 25-100 ng of

DNA was then added to 50 μ l of cells, the cells placed on ice for 2 min, incubated at 42° C for 1.5 min, then placed on ice again for 2 min. To rescue the cells, 200 μ l of R.T. SOC medium (2% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 10 mM NaCl, 2.5 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added, and the cells incubated at 37° C for 1 h with vigorous shaking. 100 μ l of cells were then plated to selective medium and the plate incubated overnight.

For strains of *E. coli* other than DH5 α and HB101 that were not routinely transformed, competent cell preparations were made and used immediately following a modification of the method of Cohen et al. (20): a saturated *E. coli* broth culture was sub-cultured to pre-warmed ML broth and incubated at 37° C with vigorous shaking until the OD₅₅₀ reached 0.5. The cells were then collected by centrifugation (7.8 kRCF_{ave}, 10 min, 4° C), the supernate drained, the cells suspended in 1 volume ice-cold 30 mM CaCl₂ solution and placed on ice for 30 min. The cells were then collected by centrifugation as above, the supernate drained, and the cells suspended in 0.1 volume of 75 mM CaCl₂. 200 μ l of these cells were then added to 25-100 ng of DNA and placed on ice for 60 min. The cells were then incubated at 42° C for 5 min, and again placed on ice for 30 min. To rescue the cells, they were mixed with 5 ml of R.T. ML broth and incubated for

2 h at 37° C with vigorous shaking. A 100 µl aliquot of this cell suspension was then plated to selective medium, and the plate incubated overnight.

Plasmid Constructions (Table 2)

pGAT400. pGAT400 is an *E. coli*-*Bacteroides* shuttle plasmid whose characteristics have been previously described by Hecht and Malamy (42). It is a fusion of the *E. coli* plasmid pDG5 (itself a fusion of the ColE1 replicon, the ampicillin resistance gene, *bla*, and the RK2 origin of transfer, *oriT_{RK2}* (37)) and the *B. fragilis* conjugative (i.e., transferable by a conjugation-type mechanism) plasmid pBFTM10 (113) (fig. 1). pGAT400 confers ampicillin resistance in *E. coli* owing to the *bla* gene, and clindamycin resistance in *Bacteroides*, due to a clindamycin resistance gene located on the resident transposon of pBFTM10, Tn4400 (81).

In pGAT400, both pDG5 and pBFTM10 have active transfer properties. Thus, when co-resident with an IncPα conjugal (i.e., self-transferable) plasmid such as RK2 or pRK231, pGAT400 can be mobilized from *E. coli* to *E. coli* (34), or to *Bacteroides* (35). This mobilization is due to the transfer proteins of pRK231 acting *in trans* upon *oriT_{RK2}* of pDG5. pBFTM10 also has a transfer region that is comprised of a *cis*-acting origin of transfer,

Table 2.--Plasmids

Plasmid	Relevant phenotypes and characteristics ^a	Source
R751	Inc ρ β , Tra ⁺ , Tmp ^r	Meyer & Shapiro (56)
F' lac	IncFI, Tra ⁺ , lac ⁺	Pasteur Institute, Paris, France
pACYC184	Mob ⁻ , Cm ^r , Tc ^r	Chang and Cohen (18)
pGEM7Zf(+)	Ap ^r	Promega Corp. Madison, WI
pBR322	Tc ^r , Ap ^r	Bolivar et al. (9)
pBR328	Mob ⁻ , Cm ^r , Tc ^r , Ap ^r	Covarrubias et al. (21)
pBR328 Δ	Mob ⁻ , Ap ^r , deletion derivative of pBR328	This study
pGAT400	(Tra ⁺), (Cc ^r), Mob ⁺ , Ap ^r	Hecht, D.W. & Malamy, M. (42)
pGAT400 Δ Bgl-II	(Tra ⁻), (Cc ^r), Mob ⁻ , Ap ^r , deletion derivative of pGAT400	Hecht, D.W. & Malamy, M. (42)
pGAT400 Δ ori	(Tra ⁻), (Cc ^r), Mob ⁻ , Ap ^r , deletion derivative of pGAT400	M. Malamy, Tufts University
pJA-11a	btgA (pBFTM10) cloned into pET11D	P.J. Arnold
pJA-223B	btgB (pBFTM10) cloned into pKK223	P.J. Arnold
pJA-7ZT	pBFTM10 oriT cloned into pGEM7Zf(+)	P.J. Arnold
pLV22a	LV22 native plasmid	This study
pTJ47-1	(Tra ⁺), Mob ⁺ , Ap ^r , pLV22a::pGAT400 Δ BglIII fusion	This study
pTJ47-3	(Tra ⁺), Mob ⁺ , Ap ^r , pLV22a::pGAT400 Δ BglIII fusion	This study
pTJins1a	Ap ^r , pLV22a ligated into the pGEM7Zf(+) EcoRI site	This study
pTJins3a	Ap ^r , pLV22a ligated into the pGEM7Zf(+) EcoRI site	This study

Table 2--continued

Plasmid	Relevant phenotypes and characteristics ^a	Source
pTJ5a	<i>Mob</i> ⁺ , <i>Tc</i> ^r , pLV22a ligated into the pACYC184 <i>Eco</i> RI site	This study
pTJ8	<i>Mob</i> ⁺ , <i>Ap</i> ^r , pLV22a ligated into the pBR328Δ <i>Eco</i> RI site	This study
pB22a	<i>Mob</i> ⁺ , <i>Ap</i> ^r , pLV22a ligated to the 3.9 kb pBR322 <i>Aat</i> II/ <i>Bam</i> HI fragment	This study
pTJ6	<i>Mob</i> ⁺ , <i>Cm</i> ^r , pLV22a ligated to the 3.8kb pACYC184 <i>Xba</i> I/ <i>Bam</i> HI fragment	This study
pTJ6XB	<i>Mob</i> ⁺ , <i>Cm</i> ^r , deletion derivative of pTJ6	This study
pTJ10	(<i>Tra</i> ⁺), (<i>Cc</i> ^r), <i>Ap</i> ^r , pLV22a <i>Mob</i> region ligated into the <i>Bgl</i> III site of pGAT400Δ <i>ori</i>	This study
pTJ12	<i>Mob</i> ⁻ , <i>Cm</i> ^r , <i>mbpA</i> deletion derivative	This study
pAC7	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pAC18	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pAC38	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pAC39	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pAC49	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pAC67	<i>Mob</i> ⁻ , <i>Tc</i> ^r , <i>Tn1000</i> mutant of pTJ5a	This study
pBR7	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study
pBR18	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study
pBR38	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study
pBR39	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study
pBR49	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study
pBR67	<i>Mob</i> ⁻ , <i>Ap</i> ^r , <i>Tn1000</i> mutant of pLV22a ligated into <i>Eco</i> RI site of pBR328Δ	This study

Table 2---continued

Plasmid	Relevant phenotypes and characteristics ^a	Source
pTJ347	<i>Mob</i> ⁺ , Cm ^r , 347 bp pLV22a <i>oriT</i> PCR fragment ligated to the 4.1 kb pACYC184 <i>Sal</i> I/ <i>Sph</i> I fragment	This study
pTJ437	<i>Mob</i> ⁺ , Cm ^r , 437 bp pLV22a <i>oriT</i> PCR fragment ligated to the 4.1 kb pACYC184 <i>Sal</i> I/ <i>Sph</i> I fragment	This study

Notes: ^a Ap^r, Cc^r, Cm^r, Tc^r and Tmp^r indicate resistance to ampicillin, clindamycin, chloramphenicol, tetracycline and trimethoprim, respectively. *Tra* refers to the ability of a plasmid to transfer by a conjugation-type mechanism, while *mob* refers to the mobilization phenotype of a *tra*⁻ plasmid when co-resident with R751 (and wild-type pLV22a, in the cases of pTJ347 and pTJ437). Parenthesis denotes phenotypes expressed only in *Bacteroides*, while no parenthesis indicates phenotypes expressed only in *E. coli*.

*oriT*_{pBFTM10}, and 2 transfer genes, *btgA* and *btgB* (41) (D.W. Hecht, personal communication). When co-resident with the IncPB conjugal plasmid R751, pGAT400 can be mobilized from *E. coli* to *E. coli* or to *Bacteroides* by the *in trans* interaction of the R751 transfer apparatus and the pBFTM10 transfer region (41). pGAT400 can also transfer from *Bacteroides* to *Bacteroides*, or to *E. coli*, through the expression of the pBFTM10 transfer region (41,42).

pGAT400·BglIII and pGAT400·ori. pGAT400·BglIII is a derivative of pGAT400 that has been deleted for the transfer genes of pBFTM10, *btgA* and *btgB*, through the elimination of the pBFTM10 *Bgl*III fragments (42). pGAT400·ori is a related plasmid in which the *cis*-acting

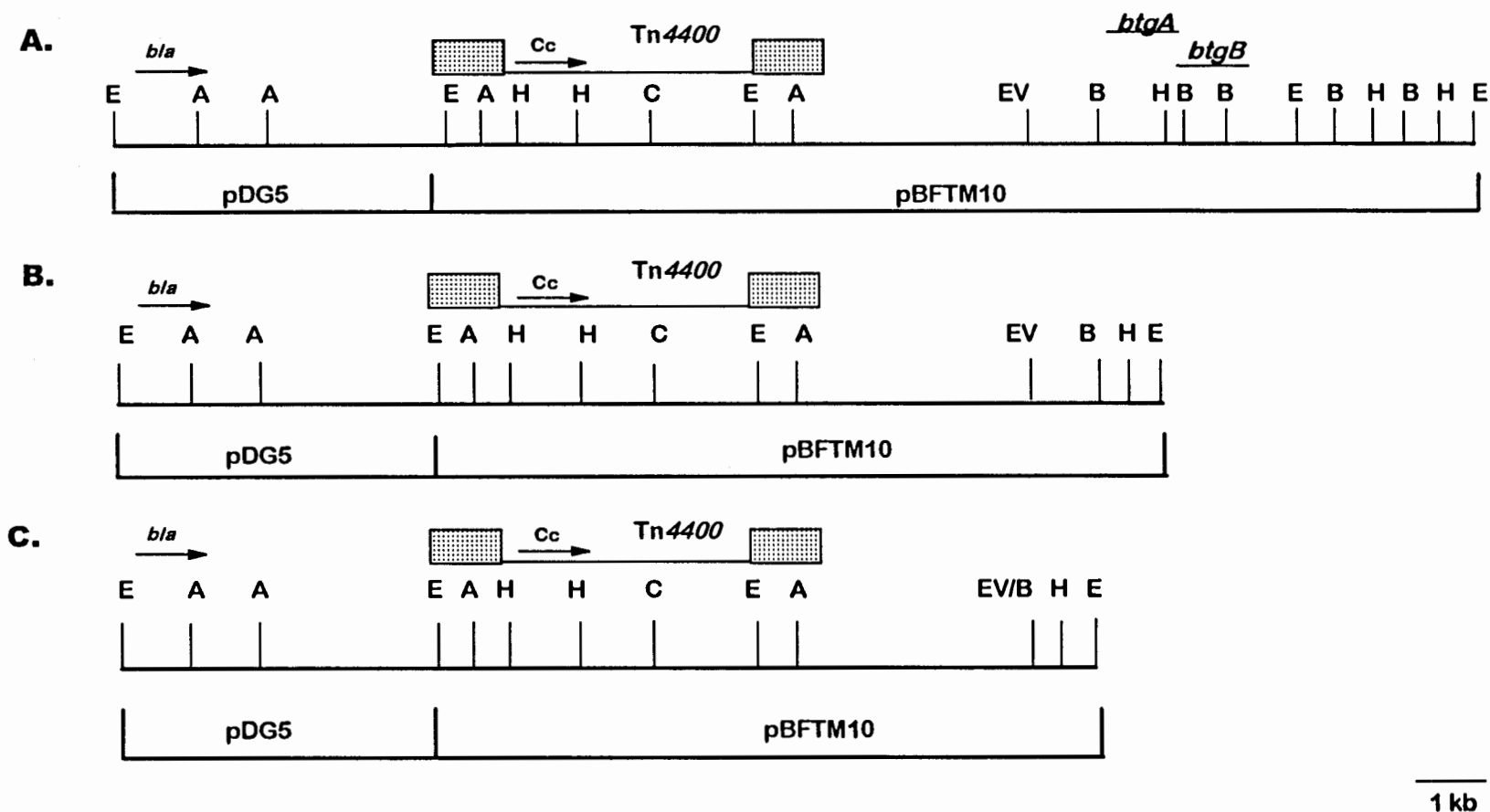


Fig. 1. Maps of (A.) pGAT400, (B.) pGAT400 *delta* BglIII and (C.) pGAT400 *delta* ori. Restriction enzymes are as follows: A, *Ava*I; B, *Bgl*III; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III. *bla* denotes ampicillin resistance gene expressed in *E. coli*; *Cc* denotes clindamycin resistance gene expressed in *Bacteroides*. Stippled boxes at the left and right ends of Tn4400 are IS4400_{left} and IS4400_{right}, respectively. *btgA* and *btgB* are transcribed from left to right in pGAT400.

oriT_{pBFTM10}, in addition to *btgA* and *btgB*, have been deleted from pGAT400 (D.W. Hecht, personal communication) (fig. 1). In contrast to their parental plasmid, pGAT400, pGAT400•BglII and pGAT400•*ori* cannot be mobilized when co-resident with R751 in *E. coli*, and are not transferable in *Bacteroides*. Like pGAT400, pGAT400•BglII and pGAT400•*ori* can still be mobilized from *E. coli* when co-resident with the IncPα plasmid pRK231, due to the *oriT_{RK2}* of the pDG5 portion of these plasmids. The replication and antibiotic resistance properties of pGAT400•BglII and pGAT400•*ori* are also identical to pGAT400.

pGEM7Zf(+), pBR322, pACYC184, and pBR328.

pGEM7Zf(+), pBR322, pACYC184 and pBR328 have been previously described (fig. 2). pACYC184 and pBR328 were chosen for use in the mating experiments because of their compatible replicons (p15A in pACYC184 and ColE1 in pBR328), and because neither pACYC184 (data not shown) nor pBR328 (21) can be mobilized when co-resident with conjugal IncP plasmids.

pBR328Δ. pBR328Δ was created for use with pACYC184 in the complementation assays by deleting the tetracycline and chloramphenicol resistance genes of pBR328. pBR328 was digested with *MscI*/*EcoRV* and the resulting 3.8 kb fragment self-ligated to give pBR328Δ (fig. 2). pBR328Δ

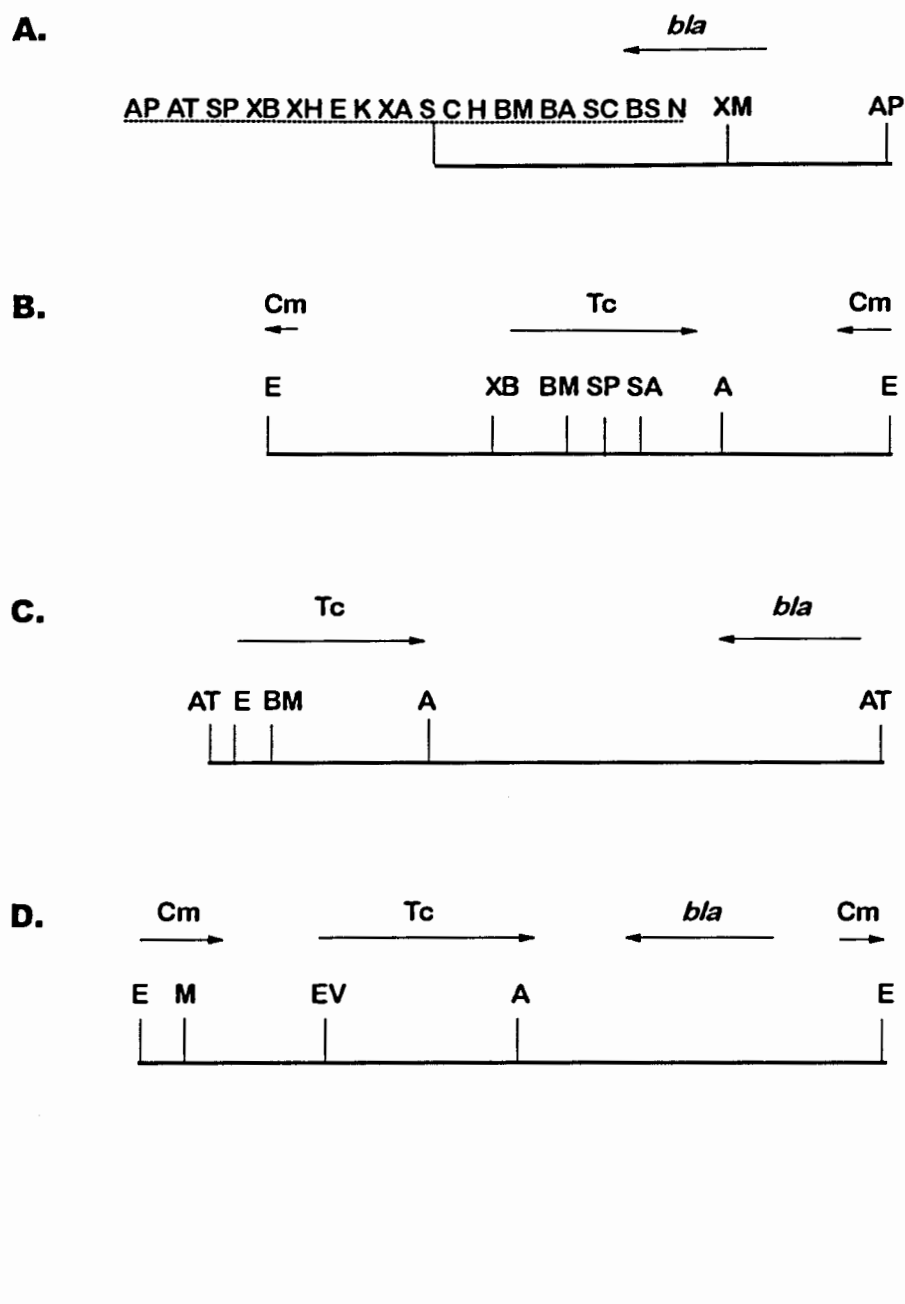


Fig 2. Maps of (A.) pGEM7Zf(+), (B.) pACYC184, (C.) pBR322, and (D.) pBR328. Restriction enzymes are as follows: A, *Ava*I; AT, *Aat*II; AP, *Apa*I; BA, *Bsa*WI; BM, *Bam*HI; BS, *Bst*XI; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; K, *Kpn*I; M, *Msc*I; N, *Nsi*I; S, *Sma*I; SA, *Sal*I; SC, *Sac*I; SP, *Sph*I; XA, *Xma*I; XB, *Xba*I; XH, *Xho*I; and XM, *Xmn*I. Dotted line indicates pGEM7Zf(+) multiple cloning site. *bla* denotes ampicillin resistance gene; *Cm* denotes chloramphenicol resistance gene; *Tc* denotes tetracycline resistance gene.

contains the pBR328 replication region and ampicillin resistance gene.

pJA-11a, pJA-223B, and pJA-7ZT. pJA-11a, pJA-223B, and pJA-7ZT were created by P.J. Arnold in the laboratory of D.W. Hecht. pJA-11a is a 6.3 kb plasmid that contains a 629 bp PCR-generated copy of the pBFTM10 transfer gene, *btgA*, ligated to the large *NcoI*/*Bam*HI fragment of pET11D. Similarly, pJA-223B is a 5.5 kb plasmid that contains a 993 bp PCR-generated copy of the pBFTM10 transfer gene, *btgB*, ligated to the large *Eco*RI/*Pst*I fragment of pKK223. Finally, pJA-7ZT contains a 304 bp PCR-generated copy of the pBFTM10 *oriT*, cloned into the *Bam*HI site of pGEM7Zf(+).

pTJins1a and pTJins3a. pTJins1a and pTJins3a were created by ligating the 5.3 kb *Eco*RI fragments of pTJ47-1 and pTJ47-3 respectively, each containing pLV22a and IS4400_{left}, into the *Eco*RI site of the pGEM7Zf(+) polylinker (figs. 3, 4).

pTJ5a and pTJ8. pTJ5a and pTJ8 were created by ligating the 5.3 kb *Eco*RI fragment of pTJ47-1 that contains pLV22a and IS4400_{left} into the unique *Eco*RI sites of pACYC184 and pBR328⁺, respectively (fig. 3).

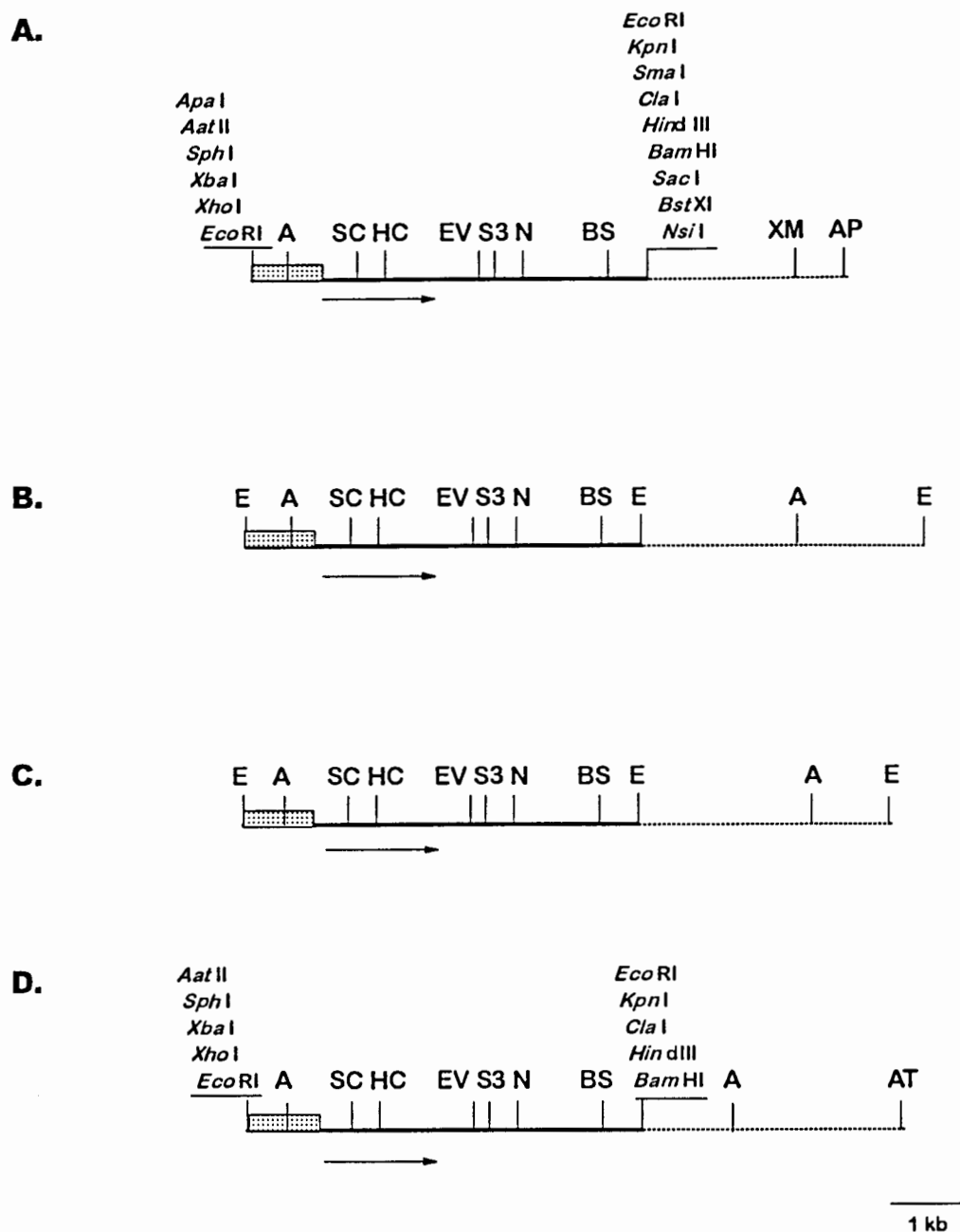


Fig. 3. Maps of (A.) pTJinsla, (B.) pTJ5a, (C.) pTJ8 and (D.) pB22a. Heavy solid line denotes pLV22a as found in pTJ47-1; dotted lines denote cloning vectors (see text for specific vectors). Stippled boxes denote IS4400_{left}. Restriction sites named in full represent sites in the pGEM7Zf(+) multiple cloning site. Other restriction sites are as follows: A, *Ava*I; AP, *Apa*I; AT, *Aat*II; BS, *Bst*XI; E, *Eco*RI; EV, *Eco*RV; HC, *Hinc*II; N, *Nsi*I; S3, *Sau*3AI; SC, *Sac*I; and XM, *Xmn*I. Arrow denotes pLV22a mobilization region.

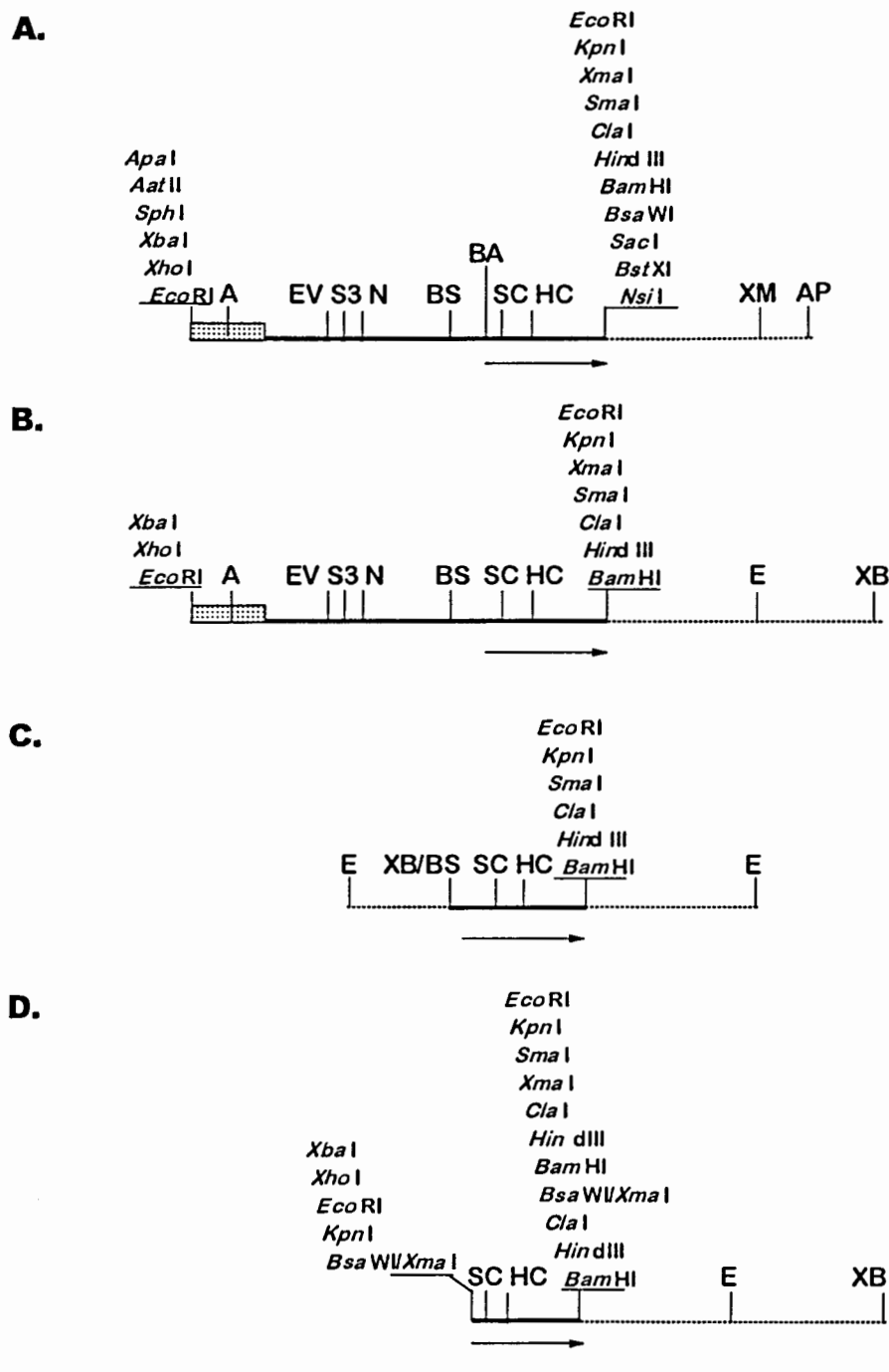


Fig. 4. Maps of (A.) pTJins3a, (B.) pTJ6, (C.) pTJ6XB and (D.) pTJ12. Heavy solid line denotes pLV22a as found in pTJ47-3; dotted lines denote cloning vectors (see text for specific vectors). Stippled boxes denote IS4400_{left}. Restriction sites named in full represent sites in the pGEM7Zf(+) multiple cloning site. Other restriction sites are as follows: A, *Ava*I; AP, *Apa*I; BA, *Bsa*WI; BS, *Bst*XI; E, *Eco*RI; EV, *Eco*RV; HC, *Hinc*II; N, *Nsi*I; S3, *Sau*3AI; SC, *Sac*I; XB, *Xba*I; and XM, *Xmn*I. Arrow denotes pLV22a mobilization region.

pB22a. pB22a was created by the ligation of the 5.3 kb AatII/BamHI fragment of pTJins1a that contains pLV22a and IS4400_{left} (from pTJ47-1) to the 3.9 kb AatII/BamHI fragment of pBR322 (fig. 3).

pTJ6. pTJ6 was created by ligation of the 5.3 kb XbaI/BamHI fragment of pTJins3a that contains pLV22a and IS4400_{left} (from pTJ47-3) to the 3.8 kb XbaI/BamHI fragment of pACYC184 (fig. 4).

pTJ6XB. pTJ6XB was created to delete IS4400_{left} and regions of pLV22a not required for mobilization. pTJ6 was first digested with XbaI/BstXI, and then with S1 nuclease to generate blunt ends. The resulting 5.5 kb fragment that contains the pLV22a mobilization region was self-ligated to give pTJ6XB (fig. 4).

pTJ10. pTJ10 was created by ligating the 3.3 kb Sau3AI/BamHI fragment of pTJ6, containing the pLV22a mobilization region, into the unique BglIII site of pGAT400•ori (fig. 5).

pTJ12. pTJ12 was developed to delete the first 32 bp of the mbpA coding region and its promoter. It was created by digesting pTJins3a with BsaWI, and then cloning the resulting 1.4 kb fragment into the XmaI site of the

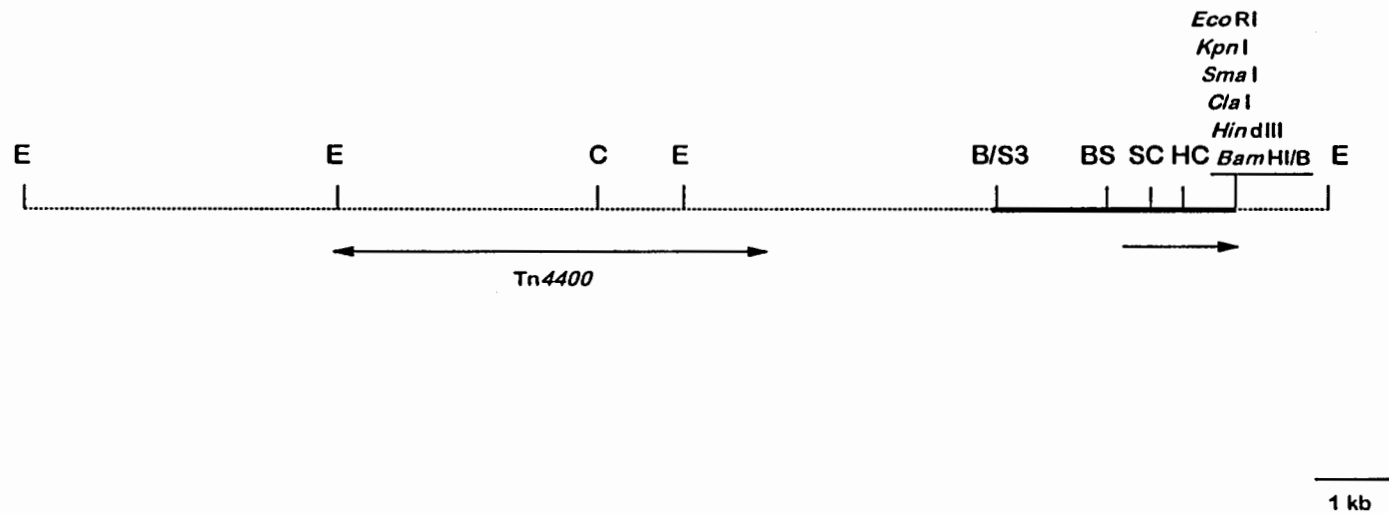


Fig. 5. Map of pTJ10. Heavy solid line denotes pLV22a DNA; dotted line represents pGAT400 *delta ori* DNA. Restriction sites named in full represent sites in the pGEM7Zf(+) multiple cloning site. Other restriction sites are as follows: B, *Bgl*II; BS, *Bst*XI; C, *Cla*I; E, *Eco*RI; HC, *Hinc*II; SC, *Sac*I; and S3, *Sau*3AI. Arrow denotes pLV22a mobilization region.

pGEM7Zf(+) polylinker. This fragment contains the pLV22a mobilization region excepting the first 32 bp of *mbpA* and its promoter. The fragment was then excised by digestion with *XbaI/BamHI*, and ligated to the 3.8 kb *XbaI/BamHI* fragment of pACYC184 (fig. 4).

Tn1000 Random Mutagenesis

Random Tn1000 insertion mutations in the pLV22a portion of pTJ5a were generated using a modification of the F plasmid mating protocol as previously described (39). A broth culture of the donor, *E. coli* DW1030 (F'*lac*)(pTJ5a), and one of the recipient, *E. coli* HB101, were grown to saturation. Into 10 ml of pre-warmed ML broth, 1 ml of donor and 2 ml of recipient were added, the broth incubated at 37° C for 1 h with vigorous shaking, and then for an additional 2.25 h with gentle shaking. The cell suspension was then serially diluted in 1X MPBS (8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 145 mM NaCl, pH 6.9), and aliquots of the dilutions plated to ML medium containing antibiotic to select for transconjugants (those recipient cells that successfully received the target plasmid), which now contained mutant target plasmids with random insertions of Tn1000. Each mutant determined to carry an insertion in the pLV22a fragment had its internal Tn1000 *BglIII* fragments deleted to prevent further transposition

of Tn1000. These mutants were designated pACinsert number, AC designating the pACYC184 replicon.

Complementation Analyses

In order to perform the complementation analysis of mutants pAC7, pAC18, pAC38, pAC39, pAC49 and pAC67, a set of plasmids containing identically placed insertion mutations was constructed. BsaW1 fragments internal to Tn1000 were deleted from pAC7, pAC18, pAC38, pAC39, pAC49 and pAC67, leaving 65 bp of Tn1000 DNA at the sites of insertion. A 5.4 kb EcoRI fragment from each of these mutants, containing pLV22a and the 65 bp of Tn1000, was then cloned into the unique EcoRI site of pBR328⁺. The resulting plasmids were designated pBR7, pBR18, pBR38, pBR39, pBR49, and pBR67 respectively, BR denoting the pBR328 replicon.

Quantitative Bacteroides to E. coli and Bacteroides to

Bacteroides Conjugative Filter Matings

To determine the transfer frequency (*F*) of conjugative plasmids and *Bacteroides* chromosomal Tet elements, quantitative *Bacteroides*-*E. coli* and *Bacteroides*-*Bacteroides* filter matings were performed as previously described (112). Broth cultures of the donor, containing the plasmid or Tet element to be assayed for transfer, and of the recipient were grown to mid-log phase

under appropriate conditions. Cultures of *Bacteroides* containing a Tet element were supplemented with 1 $\mu\text{g/ml}$ of tetracycline 4 h prior to mating. The tetracycline was then removed immediately before mating by washing the cells once with 1X MPBS (8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 145 mM NaCl, pH 6.9). (Tetracycline induction of Tet elements leads to increased transfer of both the elements themselves (74) and co-resident conjugative plasmids such as pBFTM10 (104,117).) Donor and recipient cultures were then mixed (2.5 ml:2.5 ml) in a Nalgene cellulose nitrate disposable vacuum filtration unit and the cells vacuumed unto the filter surface. The filter was then aseptically transferred to a BHIS plate, and the plate incubated anaerobically at 37° C for 18 h. The filter was then vigorously washed in 1X MPBS for 20 sec to suspend the cells. To determine the number of transconjugants/ml (those recipient cells that received the transferred plasmid or Tet element), the suspension was serially diluted in 1X MPBS, aliquots of the dilutions plated to antibiotic medium for selection of transconjugants, and the plates incubated under conditions suitable for growth of the recipient strain. The transconjugant colonies were then counted. The number of donor cells per ml in the input mid-log phase culture was also determined by serial dilution and plating. F was then calculated as:

$$\text{transconjugants/ml} \div \text{input donors/ml}.$$

Quantitative R751 Mobilization

To determine the frequency of plasmid mobilization (F_{mob}), a broth culture of the donor, *E. coli* HB101 containing R751 and the plasmid to be assayed for mobilization, and one of the recipient, *E. coli* DW1030, were grown to mid-log phase. 0.15 ml of the donor and 1.35 ml of the recipient were mixed in a 1.5 ml microcentrifuge tube, and the cells collected by centrifugation. The supernate was removed, the cells suspended in 100 μ l of 1X MPBS (8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 145 mM NaCl, pH 6.9), transferred to a 25 mm Nalgene GN-6 cellulose acetate filter supported on ML agar, and incubated for 3 hr at 37° C. The filter was then vigorously washed in 1X MPBS for 20 sec to suspend the cells. To determine the number of transconjugants/ml (those recipient cells that received R751 or the mobilized plasmid), the suspension was serially diluted in 1X MPBS. Aliquots of the dilutions were then plated to an antibiotic medium for the selection of R751 transconjugants, and also to a second antibiotic medium for the selection of mobilized plasmid transconjugants. The plates were incubated overnight at 37° C, and the transconjugant colonies counted. F_{mob} was then calculated as follows:

$$\text{transconjugants}_{mob \text{ plasmid}}/\text{ml} \div \text{transconjugants}_{R751}/\text{ml}.$$

E. coli Strain Construction Using IncP Plasmid Mobilization

Plasmids that are mobilized when co-resident with the IncP conjugal plasmids R751 or pRK231, as well as the IncP plasmids themselves, were conjugatively shuttled from one *E. coli* strain to second *E. coli* strain as follows: a saturated broth culture of the donor strain, containing R751 or pRK231 (and a mobilizable plasmid, when applicable), was mixed with an equal volume of saturated recipient broth culture. An aliquot of the mixture was plated to ML agar and incubated for 8 h at 37° C. A portion of the resulting culture was then plated to ML medium containing antibiotics to select for transconjugants (those recipient cells that received R751, pRK231 or the mobilized plasmid as desired), and the plate incubated aerobically overnight at 37° C.

Bacteroides Strain Construction Using pRK231 Plasmid Mobilization

Plasmids containing the pRK231 origin of transfer, such as pGAT400 and its derivatives, are efficiently mobilized from *E. coli* to *Bacteroides* when co-resident with pRK231. pRK231 mobilizations were performed by mixing a mid-log phase broth culture of donor *E. coli*, containing pRK231 and the plasmid to be mobilized, with an equal volume of mid-log phase *Bacteroides* recipient

culture. An aliquot of the mixture was then transferred to a BHIS plate and the plate incubated overnight aerobically at 37° C. A portion of the resulting culture was then plated to BHIS medium containing antibiotics to select for transconjugants (those recipient cells that received the mobilized plasmid), and the plate incubated anaerobically for 48 h at 37° C.

DNA Sequencing

Nucleotide sequences were determined from double stranded plasmid DNA templates using the Sequenase system (United States Biochemical) as instructed by the manufacturer. This system uses a modification of the Sanger method of sequencing DNA by enzymatic labelling of newly synthesized DNA with [$\alpha^{35}\text{S}$] dATP and di-deoxynucleotide chain termination (92). Plasmid DNA used in determining the primary wild type pLV22a sequence was prepared by Qiagen affinity column (Qiagen) or CsCl purification; mini-prep DNA was also used when sequencing mutants.

Sequencing reactions were electrophoresed in 6% Long Ranger gel (FMC Bioproducts, Inc.) in 1.2X TBE (10.7 mM Tris base, 10.7 mM boric acid, 0.24 mM EDTA, pH 8.0), with an 0.6X TBE (5.3 mM Tris base, 5.3 mM boric acid, 0.12 mM EDTA, pH 8.0) running buffer and running temperature of 47° C. An IBI (International Biotechnologies, Inc.) model

STS-45 sequencer was used. DNA sequence ladders were visualized by placing the dried gel on Kodak X-OMAT AR film, generally for 36-48 h.

By running a set of sequencing reactions for both 1.5 h and 4.0 h on the same gel, and then splicing the sequence obtained from both, we were able to regularly generate 300-350 bp of sequence per gel.

To sequence the pLV22a mobilization region, oligonucleotide primers directed outward from both ends of Tn1000 were used: 5'-TCAATAAGTTATACCAT-3' (gamma) and 5'-GAATTATCTCCTTAACG-3' (delta). Other primers, 17-20 bases in size, were synthesized as needed to fill gaps and obtain the complementary DNA sequence.

³²P-labeled DNA Probes

Supercoiled plasmid DNA, or DNA fragments previously agarose gel-purified, were labeled with [α^{32} P] dATP using the Stratagene Prime-It II random-prime labeling system as recommended by the manufacturer. Unincorporated nucleotides were separated from labeled probe DNA using a G-50 Sephadex spin column (Boehringer-Mannheim).

Southern Blot Analysis

DNA-DNA hybridization analysis was carried out using a modification of the method of Southern (111). DNA fragments to be analyzed were first separated by agarose

gel electrophoresis. The DNA was then depurinated by washing the gel slab in 0.25 M HCl for ~5 min at R.T. with gentle agitation, denatured by washing in 0.4 M NaOH and 0.6 M NaCl for 30 min at R.T. with gentle agitation, and then neutralized by washing in 0.5 M Tris pH 7.5 and 1.5 M NaCl for 30 min at R.T. with gentle agitation. The DNA was then transferred by capillary blotting or vacuum transfer to a Genescreen Plus (DuPont) nylon membrane with 10X SSC (1.5 M NaCl and 0.15 M trisodium citrate pH 7.0). The membrane was then washed in 0.4 M NaOH for 1 min at R.T. followed by a 1 m wash at R.T. with 0.2 M Tris pH 7.5 and 2X SSC (0.3 M NaCl and 0.03 M trisodium citrate pH 7.0). The filter was then dried, and placed in a glass rotator tube (Robbins Scientific), wetted with 2X SSC, and pre-hybridized with 5 ml of hybridization solution (0.9 M NaCl, 0.09 M trisodium citrate pH 7.0, 1% (w/v) sodium dodecyl sulfate, 100 µg/ml denatured salmon sperm DNA, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinylpyrrolidone and 0.2% (w/v) bovine serum albumin Fraction V) for 1 h at 65° C. ³²P-labeled DNA probe was then added to a concentration of ~1 X 10⁶ dpm/ml, and the filter incubated 4 h to 18 h at 65° C with slow rotation. The membrane was then washed twice with 2X SSC for 5 min at R.T. twice with warm 2X SSC and 1% (w/v) sodium dodecyl sulfate for 30 min at 65° C, and twice with 0.1X SSC (0.015 M NaCl and 0.0015 M trisodium citrate pH 7.0) for 30 min at R.T. The membrane

was then placed on Kodak X-OMAT AR film, generally for 18-20 h.

Polymerase Chain Reaction (PCR) Amplification of the
pLV22a *oriT*

The *ori T* of pLV22a was cloned using a Perkin-Elmer thermocycler and Core Reagent kit as recommended by the manufacturer. 36 amplification cycles, a 55° C annealing temperature and a 30 sec elongation time were used. A 347 bp product was generated using the primer pair: 5'-GCAAGAAGTGTCTTGTCGG-3' and 3'-CTCTTGCCCCGTGATTAATG-5'. A 437 bp product was generated using the primer pair: 5'-GCAAGAAGTGTCTTGTCGG-3' and 3'-GTCATGCTGGAGCGGTTTCTC-5'. The PCR products were first cloned into the PCR product cloning site of the pGEM-T vector (Promega). The products were then cut out with *SalI* and *SphI* restriction endonucleases, and ligated to the 4.1 kb *SalI/SphI* pACYC184 fragment. The final plasmids, containing the 347 bp and the 437 bp PCR products, respectively, were designated pTJ347 and pTJ437. The products were verified by DNA sequencing.

CHAPTER 4

RESULTS

Description of the Transfer Mechanisms of the *B. fragilis*

Strain LV22

LV22 is a clinically isolated strain of *B. fragilis*. An earlier study of LV22 from the laboratory of D.W. Hecht determined the following: (a) LV22 contains 2 native plasmids and a Tet Element. (Tet Elements are a family of closely related, self-transferable chromosomal elements that confer tetracycline resistance in *Bacteroides* (74,102)). (b) LV22 is unexpectedly able to restore transferability to the normally transfer-deficient (*Tra*⁻) plasmid pGAT400•BglIII. LV22 apparently does so by complementing *in trans* for *btgA* and *btgB*, the pBFTM10 transfer genes deleted from pGAT400•BglIII. (*btgA* and *btgB* have not been found in LV22 by high-stringency Southern hybridization analysis.) We have termed the mechanism responsible for transfer of pGAT400•BglIII the Trans Factor. (c) LV22 can also transfer other *Tra*⁻ plasmids *in cis*; the mechanism responsible for this has been termed the Cis Factor (data not shown). LV22 therefore appears to have three distinct mechanisms implicated in

conjugative transfer (i.e., transfer by a conjugation-type mechanism): a Tet Element, the Trans Factor, and the Cis Factor. The original intent of this dissertation project was to clone and characterize the LV22 Trans Factor.

Conjugative Transfer of the Trans Factor from *B. fragilis*
LV22 to *B. thetaiotaomicron* BT4001

Based upon their different phenotypes, we believed that the LV22 Trans and Cis Factors were not genetically linked, and could thus be separated to facilitate their characterization. Since the Trans Factor restored a transferable (*Tra*⁺) phenotype to pGAT400•BglIII, we hypothesized that it might also be capable of self-transfer, and consequently could be segregated from the Cis Factor. To test whether the Trans Factor was indeed transferable, a filter mating between LV22 and *B. thetaiotaomicron* BT4001 was performed. BT4001 was chosen since it contains no known transfer factors (101), and can not transfer pGAT400•BglIII (data not shown). Direct selection for transfer of the Trans Factor was not possible, since the Trans Factor is apparently cryptic. Based upon the possibility of unlinked co-transfer of the Trans Factor and the Tet Element, we instead used tetracycline to select for transfer of the LV22 Tet Element.

In the mating between *B. fragilis* LV22 and *B. thetaiotaomicron* BT4001, transfer of the Tet Element from LV22 to BT4001 occurred at a frequency of 7.2×10^{-6} transconjugants (those BT4001 recipients that received the Tet Element) per input donor. From these, fifty tetracycline resistant transconjugants were chosen for further characterization, and were designated BT22.1 through BT22.50 (*B. thetaiotaomicron* BT4001 X *B. fragilis* LV22).

Investigation of the Transfer Properties of *B. thetaiotaomicron* Strains BT22.1-BT22.50

Our next objective was to determine whether any of the *B. thetaiotaomicron* BT22 transconjugants contained the LV22 Trans Factor. In LV22, the Trans Factor has the ability to transfer the *Tra*⁻ plasmid pGAT400•BglIII. We therefore screened BT22.1-BT22.50 for the presence of the Trans Factor by filter mating BT22.1 (pGAT400•BglIII) - BT22.50 (pGAT400•BglIII) with *E. coli* HB101. 37 of the 50 BT22 strains transferred pGAT400•BglIII to HB101 at frequencies comparable to the transfer of pGAT400•BglIII from LV22 (data not shown). These data suggested that the 37 BT22 strains contained the LV22 Trans Factor. However, when transconjugant plasmid DNA transferred from the 37 BT22 strains was analyzed, pGAT400•BglIII was frequently found to have been altered. This was in direct contrast

to LV22, which did not alter pGAT400•BglII upon transfer (D.W. Hecht, personal communication). *Ava*I restriction endonuclease analysis of pGAT400•BglII from 44 transconjugants found that while 76% appeared unaltered, 20% displayed significant deletions of DNA, the majority of which involved the loss of a 4.4 kb *Ava*I fragment (fig. 6). (This fragment contains most of the resident transposon of pGAT400•BglII, Tn4400 (fig. 1).) In addition to the examples of unaltered or deleted plasmid DNA, restriction endonuclease analysis also identified 2 plasmids that had acquired approximately 1.2 kb of new DNA in the 4.4 kb *Ava*I fragment of pGAT400•BglII (fig. 7).

We concluded from these data that a genetic element from LV22 was present in the 37 BT22 strains now capable of transferring pGAT400•BglII. However, the differential phenotypes of pGAT400•BglII transfer observed in LV22 and the 37 BT22 strains made it difficult to determine whether this element was the Trans Factor. The appearance of 2 transconjugant plasmids containing 1.2 kb insertions of exogenous DNA was nonetheless considered significant, since we hypothesized that the insertions might contain transfer regions that were responsible for the plasmid's *Tra*⁺ phenotype. We thus decided to analyze the 2 insert-bearing plasmids further, and named them pTJ47-1 and pTJ47-3.

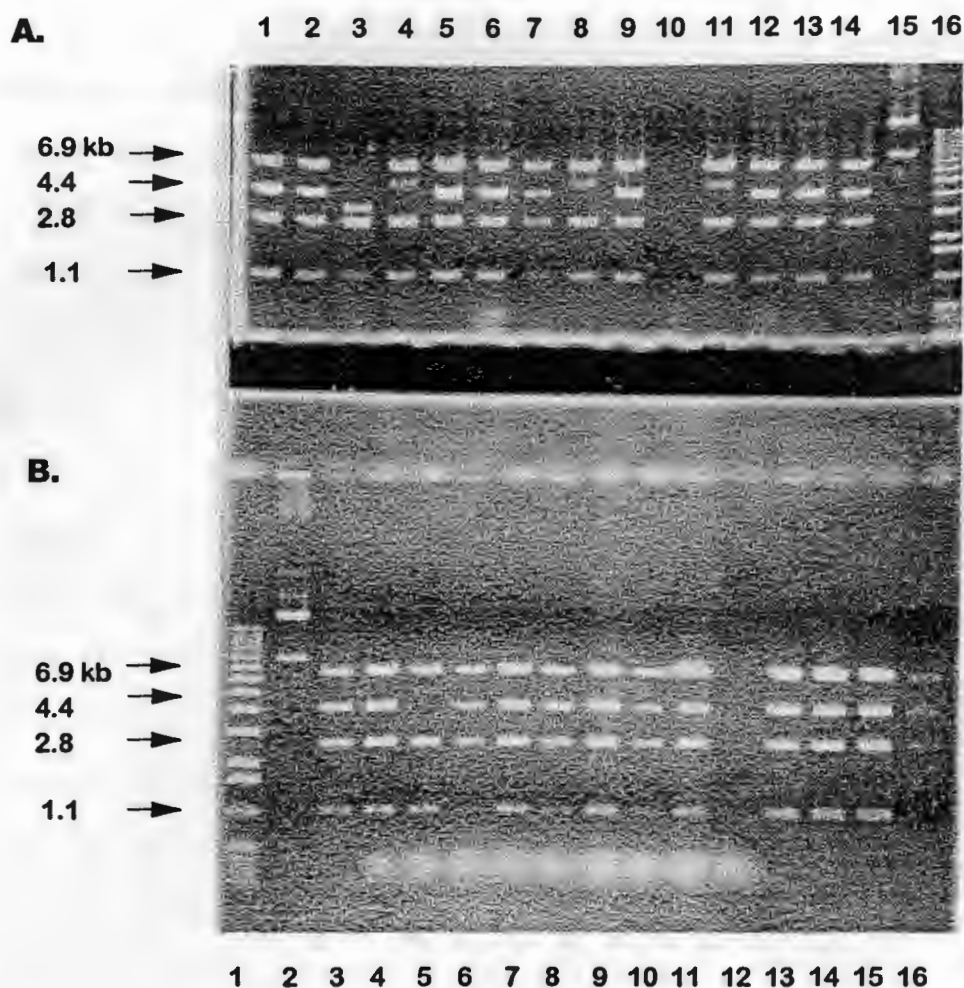


Fig. 6. *Ava*I restriction digestion patterns of *B. thetaiotaomicron* BT22.1-BT22.50 (pGAT400deltaBglII) X *E. coli* HB101 transconjugant plasmids. Gel **A**: lanes 1-13, *Ava*I digested transconjugant plasmids; lane 14, *Ava*I digested pGAT400deltaBglII; lane 15, undigested pGAT400deltaBglII; lane 16, 1 kb ladder. Gel **B**: lane 1, 1 kb ladder; lane 2, undigested pGAT400deltaBglII; lane 3, *Ava*I digested pGAT400deltaBglII; lanes 4-16, *Ava*I digested transconjugant plasmids.

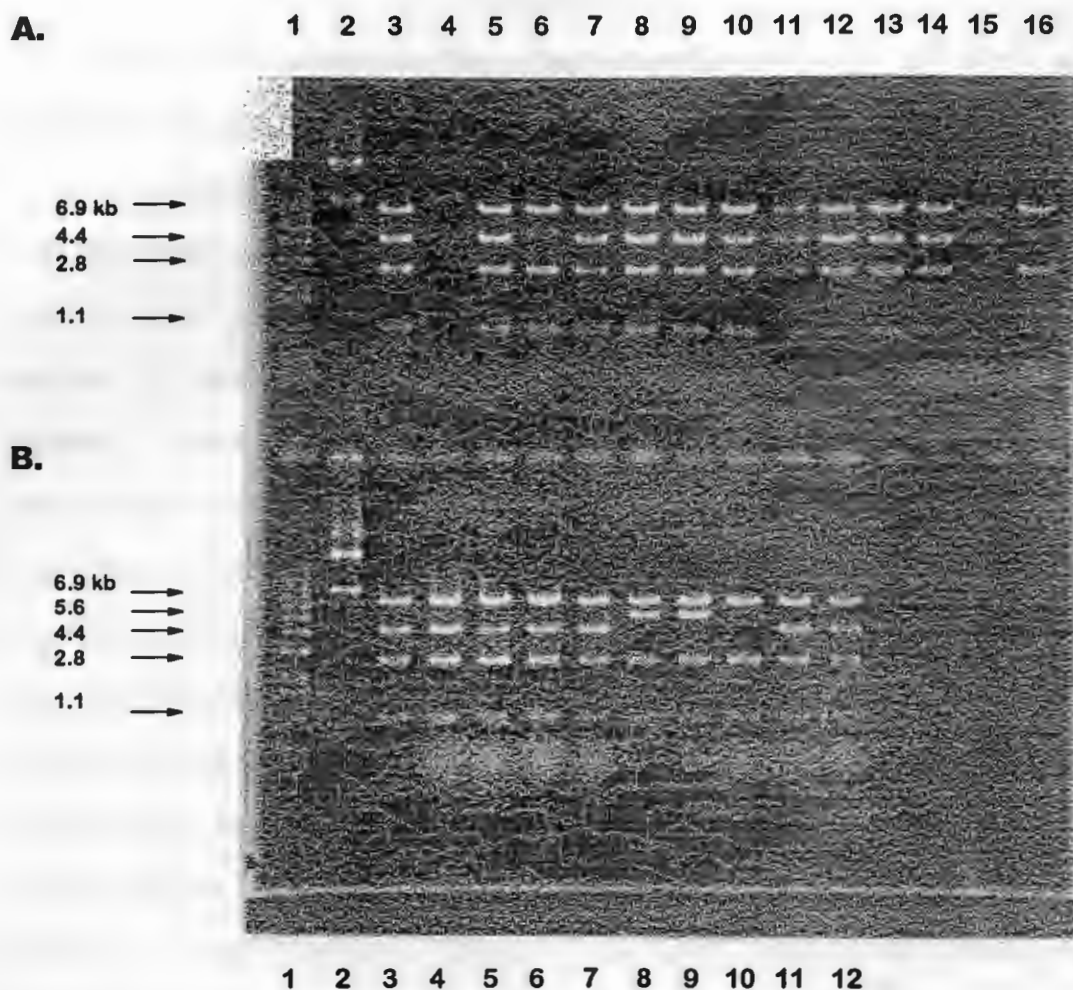


Fig. 7. *Ava*I restriction digestion patterns of pTJ47-1 and pTJ47-3. Gel A: lane 1, 1 kb ladder; lane 2, undigested pGAT400deltaBglII; lane 3, *Ava*I digested pGAT400deltaBglII; lanes 4-16, *Ava*I digested transconjugant plasmids. Gel B: lane 1, 1 kb ladder; lane 2, undigested pGAT400deltaBglII; lane 3, *Ava*I digested pGAT400deltaBglII; lanes 4-12, *Ava*I digested transconjugant plasmids. Lane 8 contains pTJ47-1; lane 9 contains pTJ47-3.

Initial Analysis of the Exogenous DNA of pTJ47-1 and
pTJ47-3

The first step in the characterization of pTJ47-1 and pTJ47-3 was to determine the locations of the new DNA within the parental plasmid, pGAT400·BglIII, using restriction endonuclease mapping. The exogenous DNA of pTJ47-1 and pTJ47-3 was initially positioned within a region of pGAT400·BglIII containing a resident transposon, Tn4400. Tn4400 consists of a core region, encoding antibiotic resistance determinants, and identical IS elements, IS4400_{left} and IS4400_{right}, which flank the core in a directly repeated orientation. The IS elements are responsible for transposition of the transposon, and both the external and internal ends of the IS elements are functional in this regard (81). Additional restriction endonuclease analysis found that while IS4400_{left} and IS4400_{right} were intact, the 3.0 kb core region of Tn4400 had been deleted (fig. 8). DNA sequencing of the junctions between the IS ends and the exogenous DNA confirmed that the core of Tn4400, up to the ends of IS4400_{left} and IS4400_{right}, had been replaced with new DNA (data not shown). Consequently, pTJ47-1 and pTJ47-3 did not carry simple 1.2 kb insertions as originally thought, but instead had simultaneously lost 3.0 kb of Tn4400 core DNA and acquired 4.2 kb of new DNA in its place.

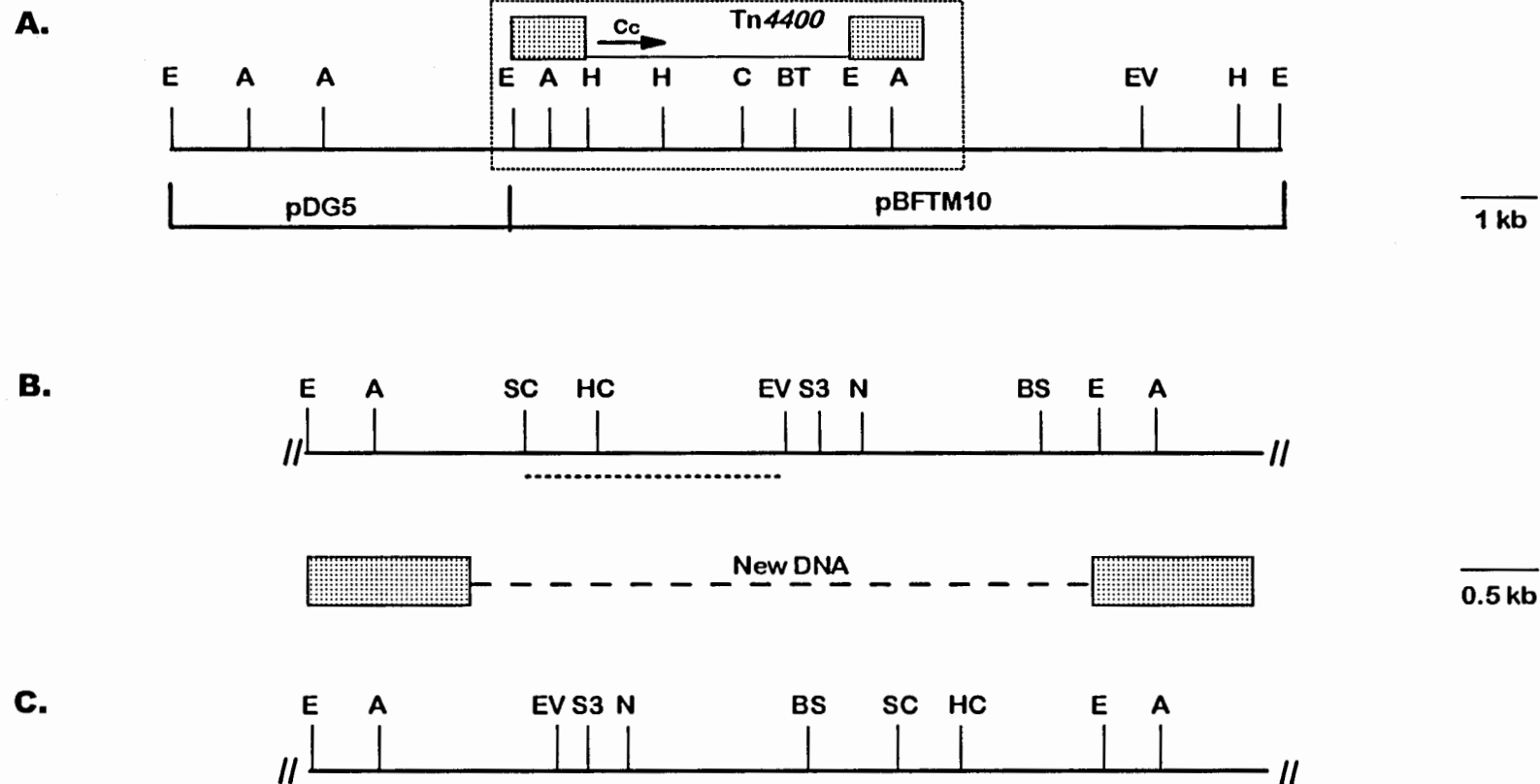


Fig. 8. Maps of (A.) pGAT400 delta BglII, (B.) pTJ47-1 and (C.) pTJ47-3. Restriction enzymes are as follows: A, *Ava*I; BS, *Bst*XI; BT, *Bst*UI; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; HC, *Hinc*II; N, *Nsi*I; S3, *Sau*3AI; and SC, *Sac*I. Stippled boxes at the left and right ends of Tn4400 are IS4400_{left} and IS4400_{right}, respectively. Section A: linear map of pGAT400 delta BglII, the parental plasmid of pTJ47-1 and pTJ47-3. The dotted box is expanded in Section B. Cc denotes clindamycin resistance determinant. Section B: linear maps of pTJ47-1 and pTJ47-3, indicating the regions of new DNA within pGAT400 delta BglII, and showing the accompanying new restriction sites. Dotted line denotes 1.8 kb *Sac*I-*Eco*RV probe fragment. // denotes truncated DNA.

Endonuclease restriction mapping of the exogenous DNA in pTJ47-1 and pTJ47-3 identified similar *Bst*XI, *Eco*RV, *Hinc*II, *Nsi*I, *Sac*I and *Sau*3AI restriction endonuclease sites in the new regions of the plasmids, although the sites in pTJ47-1 appear to be shifted with respect to the sites in pTJ47-3 (fig. 8). These data suggested that the exogenous DNA of pTJ47-1 and pTJ47-3 was an identical, 4.2 kb plasmid.

Determination of the Origin of the Exogenous DNA of
pTJ47-1 and pTJ47-3

Our next objective was to learn if the exogenous DNA of pTJ47-1 and pTJ47-3 was a 4.2 kb plasmid as we suspected, and to determine the origin of this DNA. pTJ47-1 and pTJ47-3 were formed when their parental plasmid, pGAT400·*Bgl*III, was transferred from *B. thetaiotaomicron* BT22.47 to *E. coli* HB101, and acquired new DNA in the process. BT22.47 in turn is a result of a mating between *B. fragilis* LV22 and *B. thetaiotaomicron* BT4001, and contains DNA from both parents. Thus, the new DNA of pTJ47-1 and pTJ47-3 potentially had its origins in LV22, BT4001, or both strains. We utilized high-stringency Southern hybridization analysis to determine the source and nature of the exogenous DNA. First, the 5.3 kb restriction endonuclease *Ava*I fragment of pTJ47-1, containing the exogenous DNA and IS4400_{left} (fig. 8), was

labeled with [^{32}P] and used to probe whole-cell LV22 and BT4001 DNA digested with *Bam*HI/*Sma*I, *Eco*RI, *Eco*RV and *Hind*III restriction endonucleases. The 5.4 kb *Ava*I fragment of pTJ47-1 hybridized with LV22 DNA, while no homology was exhibited with BT4001, indicating that the exogenous DNA was derived from LV22 alone (fig. 9). Furthermore, the relatively high intensity of the autoradiograph bands supported our premise that the exogenous DNA was one of two plasmids native to LV22. (A similar-sized plasmid is also present in BT22.47, but not BT4001, as seen in EtBr-stained agarose gels (data not shown).) A second Southern hybridization was then performed to determine the relationship between the exogenous DNA of pTJ47-1 and pTJ47-3, and the plasmids present in LV22 and BT22.47. Plasmids from *B. fragilis* LV22, *B. thetaiotaomicron* BT4001 and *B. thetaiotaomicron* BT22.47, and plasmids pTJins1a and pTJins3a, were digested with *Hinc*II/*Eco*RV. (pTJins1a and pTJins3a contain the exogenous DNA fragments of pTJ47-1 and pTJ47-3, respectively (Figs. 3 and 4).) The cloning vector of pTJins1a and pTJins3a, pGEM7Zf(+), was digested with *Pvu*II and included as a negative control. The resulting digests were separated by agarose gel electrophoresis, transferred to nylon membranes, and then probed with a [^{32}P]-labeled 1.8 kb *Sac*I/*Eco*RV fragment of pTJ47-1. This fragment is internal to the exogenous DNA of pTJ47-1, and overlaps the

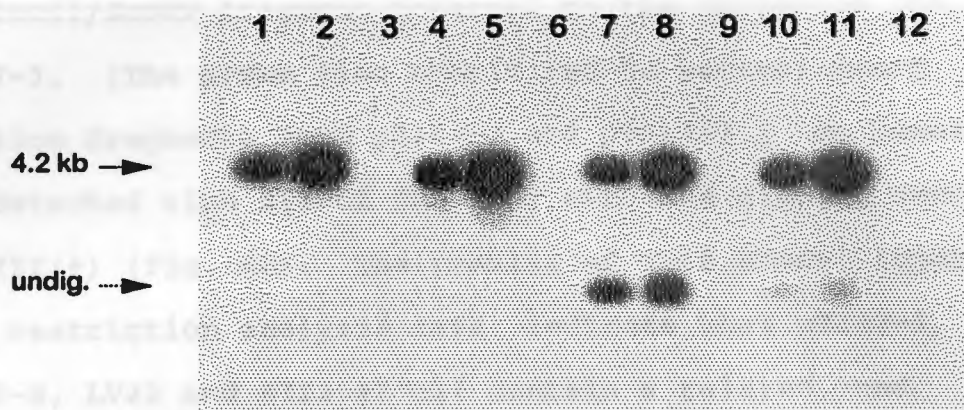


Fig. 9. Southern hybridization autoradiograph of *B. fragilis* LV22 and *B. thetaiotaomicron* BT4001 total DNA probed with the 5.4 kb *AvaI* fragment of pTJ47-1. Lane loading: 1-2, *EcoRI* digested LV22 DNA; 3, *EcoRI* digested BT4001 DNA; 4-5, *EcoRV* digested LV22 DNA; 6, *EcoRV* digested BT4001 DNA; 7-8, *BamHI/SmaI* digested LV22 DNA; 9, *BamHI/SmaI* digested BT4001 DNA; 10-11, *HindIII* digested LV22 DNA; 12, *HindIII* digested BT4001 DNA. undig. represents probable undigested DNA. Autoradiograph was exposed for 2 d.

internal *HincII*/*EcoRV* fragments of the exogenous DNA in both pTJ47-1 and pTJ47-3 (fig. 8). The probe hybridized to 1.2 kb and 3.0 kb *HincII*/*EcoRV* plasmid fragments of LV22 and BT22.47, to the 1.2 kb *HincII*/*EcoRV* fragment internal to the exogenous DNA of pTJ47-1, and to the 3.0 kb *HincII*/*EcoRV* fragment internal to the exogenous DNA of pTJ47-3. (The probe also hybridized to vector/insert junction fragments from pTJ47-1 and pTJ47-3.) No homology was detected with BT4001 DNA, nor with the cloning vector, pGEM7Zf(+) (fig. 10). The results of this study, together with restriction analysis data, indicate that pTJ47-1, pTJ47-3, LV22 and BT22.47 all contain a related, and probably identical, 4.2 kb plasmid that we have designated pLV22a.

These data indicated that pLV22a had transferred from *B. fragilis* LV22 to *B. thetaiotaomicron* BT4001 during the original mating that produced *B. thetaiotaomicron* BT22.47. We hypothesized that pLV22a might contain an active transfer region, and was therefore responsible for its own transfer into BT4001. Our next set of experiments were therefore designed to investigate what transfer properties, if any, were possessed by pLV22a.

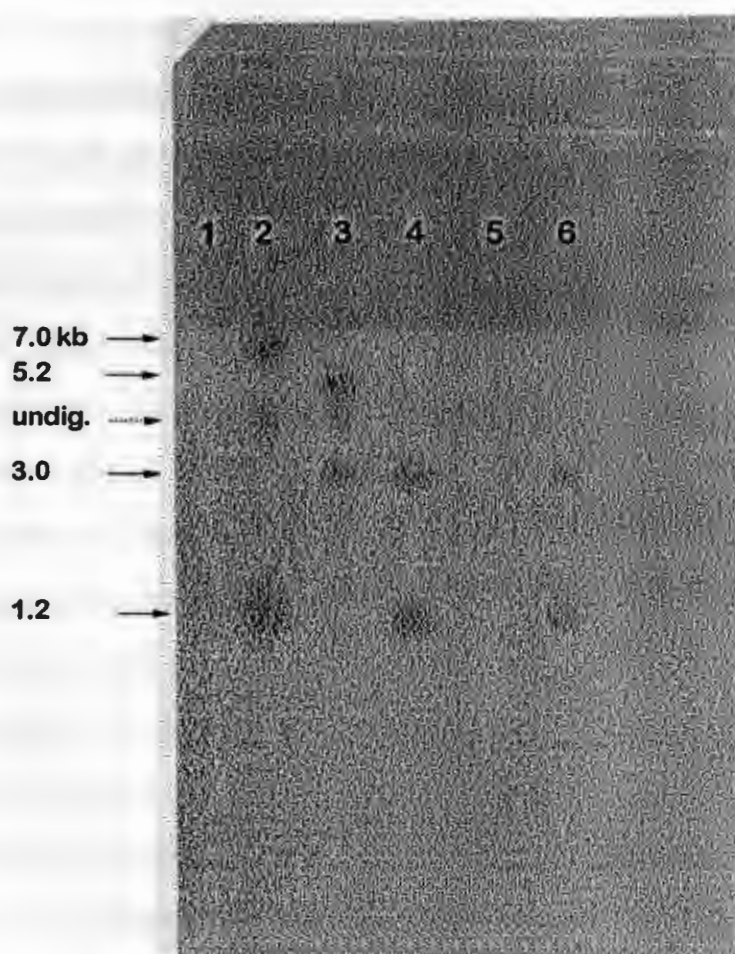


Fig. 10. Southern hybridization autoradiograph of *B. fragilis* LV22, *B. thetaiotaomicron* BT4001, and *B. thetaiotaomicron* BT22.47 plasmids probed with the 1.8 kb *SacI/EcoRV* fragment of pTJ47-1. Lane loading: 1, *PvuII* digested pGEM7Zf(+); 2, *EcoRV/HincII* digested pTJins1a; 3, *EcoRV/HincII* digested pTJins3a; 4, *EcoRV/HincII* digested plasmid contents of LV22; 5, *EcoRV/HincII* digested plasmid contents of BT4001; 6, *EcoRV/HincII* digested plasmid contents of BT22.47. undig. represents probable undigested plasmid DNA. Autoradiograph was exposed for 30 m.

Investigation of the Transfer Properties of pLV22a While
in *E. coli*

Our next line of inquiry attempted to define what transfer capabilities were possessed by pLV22a, and to thus confirm that pLV22a was responsible for the *Tra*⁺ phenotype of pTJ47-1 and pTJ47-3. pLV22a is a *B. fragilis* plasmid, and accordingly we predicted that it would be transferable from that species. However, pTJ47-1 and pTJ47-3 could not be tested for transfer from a *B. fragilis* host, since they carried no antibiotic resistance genes expressed in *Bacteroides*. (The clindamycin resistance gene of their parental plasmid, pGAT400·BglIII, had been deleted during the formation of pTJ47-1 and pTJ47-3. pTJ47-1 and pTJ47-3 thus could not be detected by antibiotic selection in *B. fragilis*. See Initial Analysis of the Exogenous DNA of pTJ47-1 and pTJ47-3, Chapter 4.) We therefore decided to initially test pLV22a for transfer in *E. coli*. This was not without precedent. The *Bacteroides* plasmids pBFTM10 and pB8-51, and the *Bacteroides* transposon Tn4399, cannot transfer from *E. coli* unassisted. However, they can all transfer from *E. coli* when co-resident with R751, a conjugal broad-host-range IncPB plasmid (i.e., they are mobilized when co-resident with R751; *Mob*⁺) (59,104,117).

To test whether pTJ47-1 and pTJ47-3 are mobilized when co-resident with R751, *E. coli* HB101 (R751) (pTJ47-1)

and HB101 (R751)(pTJ47-3) were filter mated with *E. coli* DW1030. pTJ47-1 and pTJ47-3 were mobilized at frequencies similar to that of pBFTM10 (in pGAT400), when co-resident with R751 (table 3). This was in direct contrast to the parental plasmid of pTJ47-1 and pTJ47-3, pGAT400 Δ BglIII, which was not mobilized (*Mob*⁻). Thus, the mobilization of pTJ47-1 and pTJ47-3 is directly attributable to pLV22a. However, pLV22a, like pBFTM10, pB8-51, and Tn4399, requires the co-residence of R751 for mobilization in *E. coli*.

Table 3.--R751 mobilization of pLV22a derivatives in *E. coli*

Plasmids	Presence of pLV22a in cis (+/-)	Mean frequency of mobilization ^a (\pm SEM)
pGAT400	--	2.9 (\pm 1.2) $\times 10^{-2}$
pGAT400 Δ BglIII	--	< 1.0 $\times 10^{-5}$
pACYC184	--	< 1.0 $\times 10^{-5}$
pTJ47-1	+	3.7 (\pm 1.9) $\times 10^{-3}$
pTJ47-3	+	3.9 (\pm 0.6) $\times 10^{-3}$
pTJ5a	+	3.2 (\pm 0.4) $\times 10^{-3}$
pTJ6	+	1.0 (\pm 0.5) $\times 10^{-2}$
pTJ6XB	+	7.5 (\pm 2.8) $\times 10^{-3}$

Notes: Plasmids were mobilized from *E. coli* HB101 containing R751 to *E. coli* DW1030.

^a Mobilization frequency is defined as the number of plasmid transconjugants / number of R751 transconjugants in the same mating (N=3). 1×10^{-5} defines the limit of detection.

The Mobilization of pLV22a Independent of pGAT400·BglII

We next wanted to determine whether pLV22a contained a mobilization region capable of functioning independently of pGAT400·BglII. To investigate this, the 5.3 kb *EcoRI* restriction endonuclease fragment from pTJ47-1, containing pLV22a, was ligated into the *Mob*⁻ cloning vector pACYC184 to give pTJ5a (fig. 3). Similarly, the 5.3 kb *EcoRI* restriction endonuclease fragment from pTJ47-3, containing pLV22a, was ligated into pACYC184 to give pTJ6 (fig. 3). pTJ5a and pTJ6 were then tested for mobilization when co-resident with R751 by mating *E. coli* HB101 (R751) (pTJ5a) and HB101 (R751) (pTJ6) with *E. coli* DW1030. pTJ5a and pTJ6 were both mobilized at levels comparable to the pLV22a::pGAT400·BglII fusion plasmids pTJ47-1 and pTJ47-3 (table 3). These results indicate that pLV22a provides the functions required for its mobilization, and can thus operate independently of pGAT400·BglII in this regard.

Comparison of pLV22a to the Transfer Region of pBFTM10

We wished to learn whether pLV22a was related to pBFTM10, the only other *Bacteroides* transferable plasmid characterized at the time (41). We used a study based upon high-stringency Southern hybridization analysis to determine whether pLV22a possessed homology to the pBFTM10 origin of transfer (*oriT*), or its transfer genes, *btgA* and *btgB*. pTJins1a and pTJins3a, containing pLV22a from

pTJ47-1 and pTJ47-3 respectively, were digested with *EcoRI* restriction endonuclease, the resulting fragments were separated by agarose gel electrophoresis and then transferred to 3 nylon membranes. These membranes were then probed with [³²P]-labeled plasmids containing either the pBFTM10 *oriT* (pJA-7ZT), *btgA* (pJA-11a), or *btgB* (pJA-223B). No hybridization was detected between any of these probes and the 5.3 kb *EcoRI* restriction fragments of pTJins1a and pTJins3a, containing pLV22a (data not shown). We therefore concluded from these data that no significant homology exists between the mobilization region of pLV22a and the transfer region of pBFTM10.

We had now isolated a novel *B. fragilis* transfer factor, pLV22a, for which we had direct evidence of mobilization in *E. coli*, and transfer in *B. fragilis*. Because of this, we decided to shift the focus of our study from the LV22 Trans Factor to pLV22a.

Localization of the pLV22a Mobilization Region by Tn1000

Random Insertion Mutagenesis

Our next objective was to identify the region of pLV22a required for mobilization in *E. coli* when co-resident with R751. We used the transposon Tn1000 to mutagenize pLV22a, thereby inactivating the region(s) of pLV22a necessary for mobilization. The target plasmid, pTJ5a, was mutagenized by mating *E. coli* DW1030.

(F[']lac)(pTJ5a) with *E. coli* HB101. Of 50 Tn1000 insertion mutations generated, 37 were restriction endonuclease mapped to the pLV22a portion of pTJ5a (fig. 11). Each of the 37 mutants was then further tested for mobilization from *E. coli* HB101 (R751). Ten mutants, pAC67, pAC38, pAC39, pAC7, pAC49, pAC18, pAC20, pAC11, pAC47, and pAC42 were found to be *Mob*⁻ (data not shown). Restriction endonuclease mapping revealed that the mutations of the 10 *Mob*⁻ plasmids were located in a contiguous 1.5 kb region of pLV22a (fig. 11). The other 27 mutants remained *Mob*⁺, and mapped to the remaining 2.7 kb of pLV22a.

To verify that the 1.5 kb region of pLV22a identified by Tn1000 mutagenesis was sufficient for mobilization, the region was sub-cloned, (along with 0.2 kb of upstream DNA) into the *Mob*⁻ cloning vector pACYC184, giving pTJ6XB. pTJ6XB was then tested for mobilization when co-resident with R751 by filter mating *E. coli* HB101 (R751)(pTJ6XB) with DW1030. As predicted, mobilization of pTJ6XB was comparable to the parental plasmid pTJ6 (table 3). These results indicate that the 1.5 kb region of pLV22a identified by Tn1000 mutagenesis is sufficient for mobilization from *E. coli*, when co-resident with R751.

Transfer of pLV22a from *B. fragilis*

We hypothesized that pLV22a, a *B. fragilis* plasmid, would be transferable in *B. fragilis*. To test this

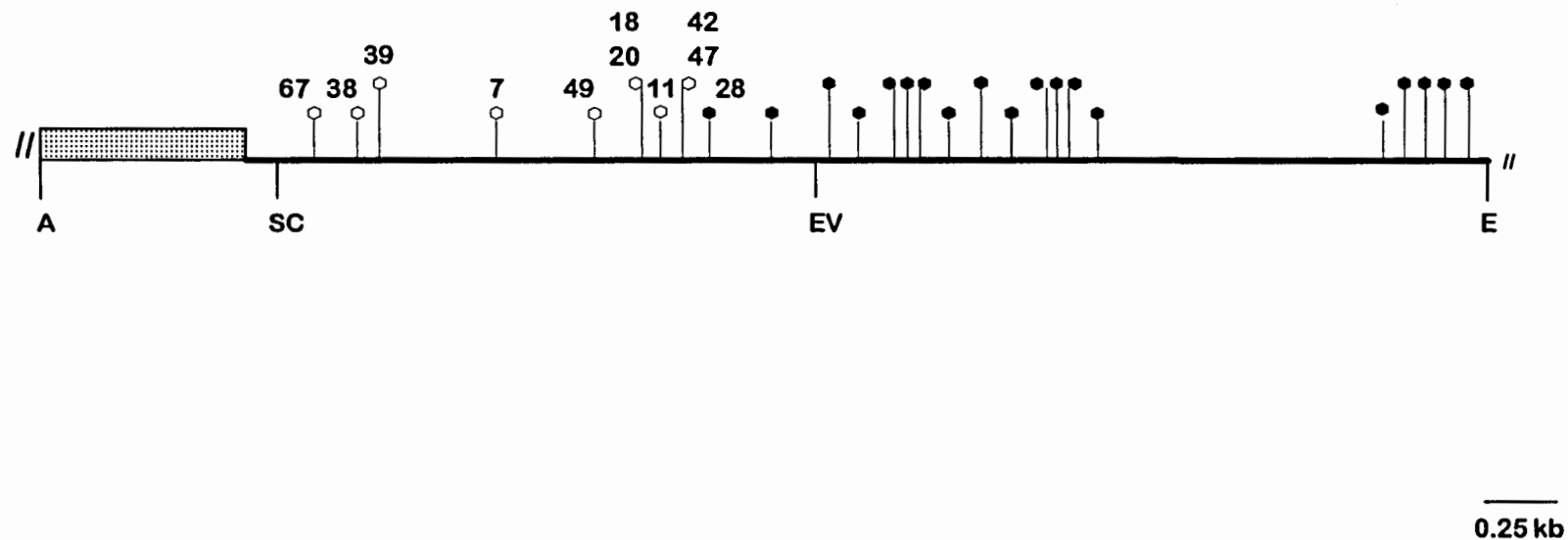


Fig. 11. Map of Tn1000 insertions in pLV22a, as found in pTJ5a. Heavy solid line denotes pLV22a DNA. // denotes truncation of vector DNA. Stippled box represents *IS4400*_{left}. Restriction sites are as follows: A, *Ava*I; E, *Eco*RI; EV, *Eco*RV; and SC, *Sac*I. Open symbols indicate mobilization-deficient Tn1000 insertions; closed symbols represent mobilization-proficient insertions. Numbers above circles indicate mutant numbers (see text).

hypothesis, a 3.3 kb *Sau3AI/BamHI* fragment containing the pLV22a mobilization region was cloned into the unique *BglIII* site of pGAT400 \cdot ori, giving pTJ10 (fig. 5).

(pGAT400 \cdot ori is a derivative of the *E. coli*-*Bacteroides* shuttle plasmid pGAT400, in which the pBFTM10 transfer region has been deleted. See Plasmid Constructions, Chapter 3.) Transfer of pTJ10 in *B. fragilis* would be attributable to the presence of pLV22a *in cis*, since pGAT400 \cdot ori is transfer-deficient. pTJ10 was tested for transfer in a mating between *B. fragilis* TM4000 (pTJ10) and *E. coli* HB101. TM4000 was selected as the donor because it is a well-characterized laboratory reference strain that is devoid of any detectable transfer factors. pTJ10 was determined to be transferable from TM4000 to *E. coli* HB101 at 7.1×10^{-9} transconjugants/input donor, a frequency comparable to that of pBFTM10 in pGAT400 (table 4). These data indicate that the pLV22a mobilization region functions at a low but measurable frequency in *B. fragilis* as well as in *E. coli*.

It has been previously shown that *Bacteroides* strains that contain Tet Elements are able to increase the transfer of co-resident plasmids such as pBFTM10 and pB8-51 (117). Tet Elements are *Bacteroides*-specific, chromosomal elements that carry tetracycline, and sometimes clindamycin, resistance genes, and are capable

Table 4.--pLV22a transfer from *B. fragilis*

Plasmids	Mean frequency of transfer ^a (\pm SEM)	
	From <i>B. fragilis</i> TM4000	From <i>B. fragilis</i> TM4.23
pGAT400	4.5 (± 0.5) $\times 10^{-9}$	8.3 (± 4.9) $\times 10^{-5}$
pGAT400 Δ ori	<1.0 $\times 10^{-9}$	<1.0 $\times 10^{-9}$
pTJ10	7.1 (± 3.0) $\times 10^{-9}$	1.5 (± 1.1) $\times 10^{-5}$

Notes: Plasmids were transferred from *B. fragilis* to *E. coli* HB101.

^a Transfer frequency is defined as the number of transconjugants/input donor in the same mating (N=3). 1×10^{-9} defines the limit of detection.

of conjugation-type transfer (4,102). The mechanism by which plasmids use the Tet Element transfer system to increase the efficiency of their own transfer is not understood, but is at least superficially similar to the mobilization of pBFTM10 and pB8-51 by R751 in *E. coli*. (See Horizontal Gene Transfer Factors, Chapter 2.)

To determine whether pLV22a transfer would be enhanced by a co-resident Tet Element, TM4.23 (pTJ10) was mated with *E. coli* HB101. (TM4.23 is a Tet Element⁺ isogeneic derivative of TM4000.) TM4.23 (pTJ10) was pre-treated with $1 \mu\text{g/ml}$ of tetracycline 2 h prior to mating, since tetracycline increases the transfer of both Tet Elements and co-resident plasmids (104,117). pTJ10 was found to transfer to HB101 at a frequency of 1.5×10^{-5} transconjugants/input donor, which represents a 3.5 order

of magnitude increase over its transfer frequency from the Tet Element⁻ isogeneic strain TM4000 (table 4). These results demonstrate that pLV22a transfer in *B. fragilis*, like pBFTM10 in pGAT400, is substantially enhanced by the co-residence of a Tet Element (table 4).

DNA Nucleotide Sequence of the pLV22a Mobilization Region

Our next course of experiments were designed to identify the pLV22a genes required for mobilization in *E. coli*. We began by sequencing the pLV22a mobilization region enzymatically by the method of Sanger. The DNA sequences of both top and bottom strands were determined using the following strategy: (a) a set of pTJ5a mutants containing Tn1000 transposon insertions located throughout the mobilization region was selected. (b) Outward-facing DNA oligonucleotide primers homologous to the ends of Tn1000 were used to generate sequence data from each mutant. (c) Additional oligonucleotide primers were used to fill gaps in the DNA sequence that occurred due to distantly spaced insertion mutations (fig. 12).

Figure
12

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1   GCGGTTGGCGTGGATGTATTCCCATGCGGCATGTGTATATATAGCAAGAA
51  GTGTCCTTGTGCGACAATTCTTGCTTTTCTCGCTTGCTCAAAAAGATTT
101  TAAGATTACCTTTGTGGCATGGAAC TAAGACGGAACGAAAAGATTACATT
      mbpA  M E L R R N E K I T F

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1051  GTCACAGCCCCGAAGCGGTACGGAGTATAAGGCAAGCGAGATAGGGAAAGG
      V T A R S G T E Y K A S E I G K G

1101  CTACACTCTCGCCCATATCGAGCGGACACAAAGCAAAGTGAAGTGCAACT
      Y T L A H I E R T Q S K L K C N

1151  CAATGAACATATCTCATGGAAAACAACTCACACCCGGAAGCGGCAGCTTC
      S M N I S H G N K L T P G S G S F
           mbpC M E T N S H P E A A A S

1201  CAACGTTAAGCAGGGACAGTACAGCCCCCGGAAAAGAAAAGATACAACGC
      G R *
      N V K Q G Q Y S P R K R K D T T

1251  CAAGGGTAAACCCCGCAGCTTGCGGTAGAACTGGTGTATCAAGAACTAAAG
      P R V N P Q L A V E L V Y Q E L K
                        49▼

1301  CGTGTGTAAGTCTATACGAAGCGCATAGAGGACGCAACAGCCCGAAAGGT
      R V E V Y T K R I E D A T A R K V

1351  ACAAATAGACGGGAAAAGCCTTGAAAGTGCCGAAAATCGCCTAAAAAACG
      Q I D G K S L E S A E N R L K N

1401  TGTGCGGACTTTGAACGGCAGGGGTACAGAATGAAAAACGGCGGTAT
      V L A D F E R Q G Y R M K N G G Y

1451  GTGGACAAACGAATTTCTGTTCTACTCAATCCTTTGTGCTGTTATCTCCCT
      V D K R I S F Y S I L C A V I S L
                        20▼ ▼18

1501  ATTGTTGCTTGTGTTTCATGTGCTATCTTTGGACGGATGCAGCCAAAGACC
      L F A C F M C Y L W T D A A K D

1551  GGGACAACACTACAAGCAGTATTATGAATACTACCAAGAGCAGGCAAGGGAA
      R D N Y K Q Y Y E Y Y Q E Q A R E
           11▼ ▼42/47

1601  CAAAAGGGGAATAAATAAACATCTAACTTTACGAATTATGGGAACAACGG
      Q K G N K *
           Φ 28▼

1651  AACACGAAGAACCCCGGTTCTTCTCATACTGAACAAGGGTGCAGTCC

1701  GGCGGCGAAATCACCCATGCCGTCCTAAACGGTAGTATCG 1740

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Fig. 12. DNA nucleotide (top line) and deduced protein (bottom line) sequences of the pLV22a mobilization region. ▼ denotes Tn1000 insertion sites, given by number (see text). Inverted repeat sequences IR1 and IR2 are indicated by horizontal arrows above DNA sequence. Q denotes the insertion site of pGAT400ΔBglII into pLV22a, as found in pTJ47-1 (IS4400_{left} ends to the left of nt 101 in pTJ47-1). Φ denotes the insertion site of pGAT400ΔBglII into pLV22a, as found in pTJ47-3 (IS4400_{right} begins to the right of nt 1608 in pTJ47-3). Primers used to generate the PCR products of pTJ347 and pTJ437 start at: ① top strand, pTJ347 and pTJ437; ② bottom strand, pTJ347; ③ bottom strand, pTJ437. * denotes translational stop codons.

Computer Analysis of the pLV22a Mobilization Region DNA Sequence

The primary DNA structure of the pLV22a mobilization region was analyzed for the presence of open reading frames (ORFs) with the DNASIS program (version 3.00 for IBM compatible personal computers, Hitachi Software Engineering Co.). Based on methionine start sites, 5 open reading frames (ORFs) of greater than 10 kDa were identified (fig. 13). *orf 1*, *orf 2* and *orf 3* are located in the top strand. *orf 1* codes for a theoretical protein product of 99 amino acids (aa) (11.7 kDa); *orf 2* codes for a theoretical 264 aa protein (29.4 kDa); and *orf 3* codes for a theoretical 150 aa protein (17.6 kDa). *orf 1* and *orf 3* are both in the first reading frame of the top strand, while *orf 2* is in the third reading frame. *orf 1* and *orf 2* overlap by 4 bp, while *orf 2* and *orf 3* overlap by 44 bp. The bottom strand of the pLV22a mobilization region contains 2 ORFs, *orf 4* and *orf 5*. *orf 4* codes for a theoretical protein product of 170 aa (18.9 kDa), while *orf 5* codes for a theoretical protein product of 119 aa (13.4 kDa). *orf 4* is in the second reading frame of the bottom strand, while *orf 5* is in third reading frame. *orf 4* and *orf 5* overlap by 326 bp. Numerous ORFs less than 10 kDa in size were also identified (data not shown).

All 10 of the *Mob⁻ Tn1000* insertion mutants of pLV22a disrupt one or more of the 5 mobilization region ORFs

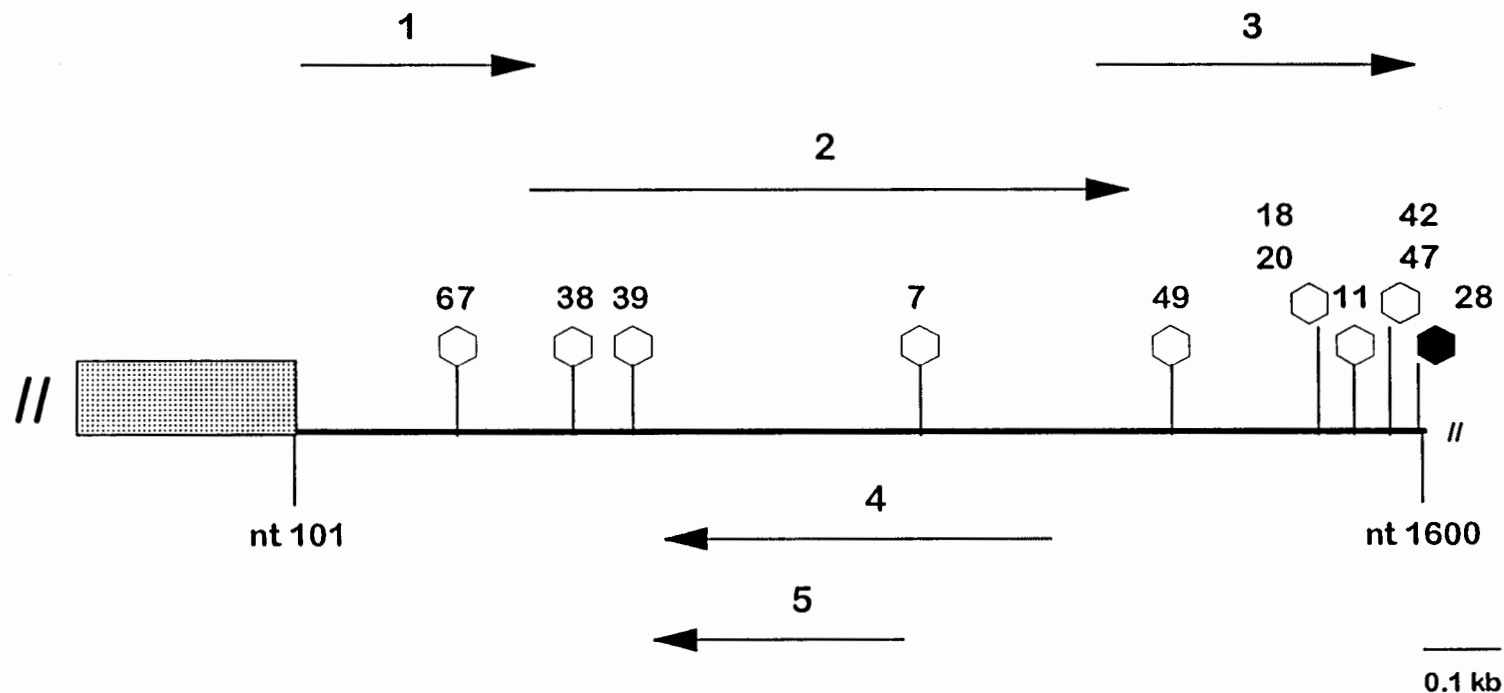


Fig. 13. Major open reading frames of the pLV22a mobilization region. Solid horizontal line represents pLV22a mobilization region, as found in pTJ47-1. Stippled box represents the right end of IS4400left. // denotes truncated DNA. Open symbols indicate mobilization-deficient Tn1000 insertions, while the closed symbol denotes a mobilization-proficient insertion; numbers correspond to mutant number. Nucleotide numbering corresponds to that of pLV22a sequence as found in Figure 12. Horizontal arrows numbered 1-5 denote open reading frames of the same number.

identified by DNA sequence analysis (fig. 13). Of these *Mob*⁻ mutants, 6 were selected for further study. The mutation of pAC67 disrupts *orf* 1, the mutations of pAC38, pAC39 and pAC7 disrupt *orf* 2, and the mutations of pAC49 and pAC18 disrupt *orf* 3. The *Mob*⁻ phenotypes of these mutants indicate that *orf* 1, *orf* 2, and *orf* 3 are required for mobilization in *E. coli* when co-resident with R751. The mutation of pAC7 also disrupts *orf* 4 and *orf* 5 of the bottom strand. Thus, *orf* 4 and/or *orf* 5 may also be required for mobilization.

pTJ47-1 and pTJ47-3 were formed when the parental plasmid, pGAT400·BglIII, became inserted into 2 different sites of pLV22a (See Initial Analysis of the Exogenous DNA of pTJ47-1 and pTJ47-3, this chapter.) Sequence analysis discovered that *orf* 3 in pTJ47-3 was interrupted 7 nucleotides upstream of the translational stop codon by the insertion of pGAT400·BglIII (fig. 12). In pTJ47-1, *orf* 3 is intact due to the alternate site of insertion into pLV22a.

Mutations in *orf* 1, *orf* 2, and *orf* 3 can be Complemented in trans

Based upon Tn1000 mutagenesis data, we concluded that *orf* 1, *orf* 2, and *orf* 3 are all required for mobilization in *E. coli*. We next used complementation analysis to demonstrate that *orf* 1, *orf* 2, and *orf* 3 produce trans-

acting products. Complementation analysis is an *in vivo* procedure that is performed by placing a mutant sequence and a wild type sequence, each on a different and compatible plasmid, together in a test cell line or strain; the strain is then analyzed for a restoration of the function that had been lost in the mutant. A restoration of function indicates that the wild type sequence provided a *trans*-acting product (e.g., a protein) that complemented the product no longer produced in the mutant. A refinement of this procedure places 2 different mutant sequences together *in trans*, after which gain of function is measured. In this case, a restoration of function to the mutants implies that their mutations are in different and independently expressed genes, or are in genes located within different operons.

pLV22a *Mob*⁻ Tn1000 mutants were first tested for the restoration of mobility when co-resident with wild-type pLV22a (and R751) *in trans*. *E. coli* HB101 (R751) strains, containing pB22a (i.e., wild-type pLV22a) and either pAC67 (i.e., a mutant of *orf* 1); pAC38, pAC39, pAC7 (i.e., mutants of *orf* 2); pAC49 or pAC18 (i.e., mutants of *orf* 3) were mated with *E. coli* DW1030 (fig. 13). pAC67, pAC38, pAC39, pAC7, pAC49 and pAC18 were all efficiently mobilized when wild-type pLV22a was provided *in trans* (table 5).

Table 5--R751 mobilization of Tn1000 insertion mutants in *E. coli*

Plasmids	Presence of pLV22a <i>in trans</i> (+/--)	Mean Frequency of Mobilization ^a (±SEM)	
<u>Wild-type</u>			
pTJ5a	NA	3.2 (±0.4)	X 10 ⁻³
pACYC184	+	< 1.0	X 10 ⁻⁵
<u>Mutants of <i>mbpC</i></u>			
pAC49	--	< 1.0	X 10 ⁻⁵
pAC49	+	1.0 (± 0.8)	X 10 ⁻²
pAC18	--	< 1.0	X 10 ⁻⁵
pAC18	+	6.6 (± 2.9)	X 10 ⁻³
<u>Mutants of <i>mbpB</i></u>			
pAC38	--	< 1.0	X 10 ⁻⁵
pAC38	+	3.8 (± 1.4)	X 10 ⁻³
pAC39	--	< 1.0	X 10 ⁻⁵
pAC39	+	9.0 (± 6.5)	X 10 ⁻³
pAC7	--	< 1.0	X 10 ⁻⁵
pAC7	+	6.2 (± 3.0)	X 10 ⁻³
<u>Mutant of <i>mbpA</i></u>			
pAC67	--	< 1.0	X 10 ⁻⁵
pAC67	+	2.7 (± 1.1)	X 10 ⁻³

Notes: Wild type pLV22a was provided *in trans*, where indicated, as part of the plasmid pB22a. Plasmids were mobilized from *E. coli* HB101 containing R751 (and pB22a, as noted) to *E. coli* DW1030. NA = not applicable.

^a Mobilization frequency is defined as the number of plasmid transconjugants / number of R751 transconjugants in the same mating (N=3). 1X10⁻⁵ defines the limit of detection.

To ensure that the mutant plasmids had not been mobilized by a *cis*-acting mechanism (e.g., cointegrate fusion with the *Tra*⁺ plasmid R751 or the *Mob*⁺ wild-type plasmid pB22a), 5 samples of transconjugant plasmid DNA from each mating were analyzed by *Bam*HI restriction endonuclease digestion. The mobilized mutant plasmids were found to be unaltered in all cases, supporting the premise that they had been mobilized *in trans* (data not shown). These results demonstrated that *orf* 1, *orf* 2, and *orf* 3 of the pLV22a mobilization region produce *trans*-acting products which are required for mobilization in *E. coli*, when co-resident with R751. Consequently, we tentatively designated *orf* 1, *orf* 2, and *orf* 3 as *mbpA*, *mbpB* and *mbpC*, respectively, for mobilization of a *Bacteroides* plasmid.

We next used complementation analysis to confirm that *mbpA*, *mbpB* and *mbpC* produce *trans*-acting products required for pLV22a mobilization, and to determine the genetic organization of *mbpA*, *mbpB* and *mbpC*. This experiment used a series of pLV22a *Mob*⁻ Tn1000 insertion mutants cloned into both pBR328⁺ (those plasmids with a BR prefix) and pACYC184 (those plasmids using an AC prefix): mutation 67 (pBR67 and pAC67) in *mbpA*; mutation 38 (pBR38 and pAC38), mutation 39 (pBR39 and pAC39) and mutation 7 (pBR7 and pAC7) in *mbpB*; mutation 49 (pBR49 and pAC49) and mutation 18 (pBR18 and pAC18) in *mbpC* (fig. 13). *E. coli* HB101

(R751) complementation strains containing both a pBR mutant plasmid and a pAC mutant plasmid were then mated with *E. coli* DW1030, and the mobilization frequencies of the pAC plasmid calculated. The mobilization of a pAC plasmid would indicate that its mutation and the mutation of its co-resident pBR plasmid were in different, independently expressed genes. Conversely, the lack of pAC plasmid mobilization would indicate that its mutation and that of its co-resident pBR mutant were located in the same gene, or in 2 genes forming an operon.

Complementation analysis of mutations in *mbpC*. The coding region of the putative mobilization gene *mbpC* extends from nucleotide (nt) 1166 to nt 1618 in the pLV22a mobilization region sequence (fig. 12), and codes for a theoretical 17.6 kDa protein. Two *Tn1000* insertion mutations were generated in *mbpC*: the mutation of pAC49 occurs at nt 1279, while that of pAC18 occurs at nt 1498 (fig. 12). Both pAC49 and pAC18 are *Mob*⁻, but can be mobilized from *E. coli* when wild-type pLV22a is present *in trans* (see Mutations in *orf 1*, *orf 2*, and *orf 3* can be Complemented in trans, this chapter). To confirm that they disrupt the same gene, both mutations were analyzed for complementation by mating *E. coli* HB101 (R751)(pAC49)(pBR18) and *E. coli* HB101 (R751)(pAC18)(pBR49) with *E. coli* DW1030. In these

matings, no transfer of the pAC plasmids to DW1030 was detected (table 6). The failure of the mutations in pAC49 and pAC18 to complement one another supports the premise that *mbpC* is a gene whose *trans*-acting protein product is required for pLV22a mobilization when co-resident with R751.

Complementation analysis of mutations in *mbpB*. The coding region of the putative mobilization gene *mbpB* extends from nt 415 to nt 1209, and codes for a theoretical 29.4 kDa protein (fig. 12). *mbpB* is interrupted by the Tn1000 insertion mutations of 3 plasmids, pAC38 (nt 478), pAC39 (nt 535) and pAC7 (nt 927) (fig. 12). All 3 are *Mob⁻*, but can be mobilized when wild-type pLV22a is present *in trans* (see Mutations in *orf* 1, *orf* 2, and *orf* 3 can be Complemented *in trans*, this chapter). To confirm that they disrupt *mbpB*, these mutations were analyzed for complementation by performing the following filter matings: *E. coli* HB101 (R751)(pAC38)(pBR39), HB101 (R751)(pAC38)(pBR7), HB101 (R751)(pAC39)(pBR38), HB101 (R751)(pAC39)(pBR7), HB101 (R751)(pAC7)(pBR38) and HB101 (R751)(pAC7)(pBR39) mated with *E. coli* DW1030. In each case, no mobilization of a pAC plasmid to DW1030 was observed (table 6). Therefore, the mutations of pAC38, pAC39 and pAC7 cannot complement one another, demonstrating that the ORF they disrupt,

Table 6.--Complementation analyses of pLV22a Tn1000 mutants in *E. coli*

Mobilization genotype	Complementing Plasmid ^a	Mobilized Plasmid ^a	Mean frequency of mobilization ^b (± SEM)	
Wild-type				
	--	pTJ5a	3.2 (±0.4)	X 10 ⁻³
	--	pACYC184	<1.0	X 10 ⁻⁵
<i>mbpC</i> (orf 3)				
	pBR49 (<i>mbpC</i> ⁻)	pAC18 (<i>mbpC</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR18 (<i>mbpC</i> ⁻)	pAC49 (<i>mbpC</i> ⁻)	<1.0	X 10 ⁻⁵
<i>mbpB</i> (orf 2)				
	pBR7 (<i>mbpB</i> ⁻)	pAC38 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR38 (<i>mbpB</i> ⁻)	pAC7 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR7 (<i>mbpB</i> ⁻)	pAC39 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR39 (<i>mbpB</i> ⁻)	pAC7 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR38 (<i>mbpB</i> ⁻)	pAC39 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR39 (<i>mbpB</i> ⁻)	pAC38 (<i>mbpB</i> ⁻)	<1.0	X 10 ⁻⁵
	pBR18 (<i>mbpC</i> ⁻)	pAC39 (<i>mbpB</i> ⁻)	2.5 (±1.4)	X 10 ⁻³
	pBR39 (<i>mbpB</i> ⁻)	pAC18 (<i>mbpC</i> ⁻)	4.7 (±2.0)	X 10 ⁻³
<i>mbpA</i> (orf 1)				
	pBR67 (<i>mbpA</i> ⁻)	pAC39 (<i>mbpB</i> ⁻)	1.1 (±0.4)	X 10 ⁻³
	pBR39 (<i>mbpB</i> ⁻)	pAC67 (<i>mbpA</i> ⁻)	9.3 (±5.8)	X 10 ⁻⁵
	pBR67 (<i>mbpA</i> ⁻)	pAC18 (<i>mbpC</i> ⁻)	1.9 (±0.8)	X 10 ⁻³
	pBR18 (<i>mbpC</i> ⁻)	pAC67 (<i>mbpA</i> ⁻)	2.2 (±1.3)	X 10 ⁻⁴

Notes: Plasmids were mobilized from *E. coli* HB101, containing R751 and a complementing plasmid where indicated, to *E. coli* DW1030.

^a See figure 13 for insertion mutation sites within *mbpA*, *mbpB*, and *mbpC*. (The number of each pBR and pAC plasmid corresponds to the mutation contained within each plasmid.)

^b Mobilization frequency is defined as the number of plasmid transconjugants / number of R751 transconjugants in the same mating (N=3). 1×10^{-5} defines the limit of detection.

mbpB, produces a trans-acting product required for pLV22a mobilization.

A Tn1000 insertion mutation in an upstream gene of an operon will theoretically stop the expression of downstream genes in that operon as well. This effect on downstream genes is known as polarity (5). Thus, the Tn1000 insertion mutations that disrupt *mbpB* could also be polar on *mbpC*, if they constitute an operon (fig. 13). To determine whether *mbpB* and *mbpC* are part of an operon, the Tn1000 insertion mutants pAC39 (*mbpB*) and pAC18 (*mbpC*) were paired with the mutants pBR18 (*mbpC*) and pBR39 (*mbpB*), respectively, in the following matings: *E. coli* HB101 (R751)(pAC39)(pBR18) and HB101 (R751)(pAC18)(pBR39) mated with *E. coli* DW1030. pBR18 fully complemented pAC39, and pBR39 fully complemented pAC18, demonstrating that *mbpB* and *mbpC* are independently promoted genes, and are not part of an operon (table 6).

Complementation analysis of a mutation in *mbpA*. The coding region of the putative mobilization gene *mbpA* extends from nt 119 to nt 418, and codes for a theoretical 11.7 kDa protein (fig. 12). *mbpA* is interrupted by the Tn1000 insertion mutation of pAC67 (nt 317). pAC67 is *Mob*⁻, but can be mobilized when wild-type pLV22a is present *in trans* (see Mutations in orf 1, orf 2, and orf 3

can be Complemented in trans, this chapter). Since *mbpA* is the only major ORF disturbed by the mutation of pAC67, and because it can be complemented by wild-type pLV22a in *trans*, we conclude that *mbpA* is a gene whose *trans*-acting product is required for pLV22a mobilization.

To test whether *mbpA* and *mbpB* are part of an operon, the following *E. coli* strains were filter-mated with *E. coli* DW1030: HB101 (R751)(pAC67)(pBR39) and HB101 (R751)(pAC39)(pBR67). Similarly, to test whether *mbpA* and *mbpC* are part of an operon, the following strains were also mated with *E. coli* DW1030: HB101 (R751)(pAC67)(pBR18) and HB101 (R751)(pAC18)(pBR67). pBR67 (*mbpA*) was able to fully complement the mutants pAC39 (*mbpB*) and pAC18 (*mbpC*). However, in the reverse experiments, pBR39 (*mbpB*) and pBR18 (*mbpC*) only partially restored mobility to the mutant plasmid, pAC67 (*mbpA*) (table 6). pBR39 and pBR18 should produce normal levels of MbpA, since their mutations are well downstream of *mbpA*. We therefore reasoned that the partial complementation of pAC67 was due to the Tn1000 insertion within *mbpA* of pAC67 itself (see following section). We concluded, however, that *mbpB* and *mbpC* do not form an operon with *mbpA*, since pBR67 fully complemented mutants of *mbpB* (pAC39) and *mbpC* (pAC18).

Localization of a cis-acting Region Required for the
Mobilization of pLV22a

Complementation analysis of pAC67, a Tn1000 insertion mutant of *mbpA*, found that while pAC67 could restore mobility *in trans* to mutants of the downstream genes *mbpB* and *mbpC*, these same mutant plasmids could only partially restore mobility to pAC67 (see Complementation Analysis of a Mutation in *mbpA*, this chapter). These data were somewhat puzzling upon initial examination. However, further analysis suggested that the Tn1000 insertion of pAC67 not only disrupts the *trans*-acting component required for mobilization, *mbpA*, but might also interfere with a *cis*-acting component of mobilization, the *oriT* (for more on the *oriT*, see Horizontal Gene Transfer Factors, Chapter 2.) When this region of DNA was analyzed for the presence of grouped direct and/or inverted repeats (DNASIS, version 3.00, Hitachi Software Engineering Co.), we identified 2 sets of adjacent imperfect inverted repeats that are 14 bp (nt 234 - nt 260) and 9 bp (nt 267 - nt 292) in size (fig. 12). Inverted repeats have been previously identified in the *oriT* sequences of several transferable plasmids, including pBFTM10 and RP4 (41,68,126). Their presence within *mbpA* supported our hypothesis that the mutation of pAC67 interferes with a *cis*-acting *oriT* that is located within the coding region of the gene.

To test this hypothesis, the pLV22a 1.5 kb mobilization region, from which the first 32 bp of the *mbpA* coding region and upstream promoter sequence had been deleted (nt 1 - nt 150, fig. 12), was cloned into the *Mob*⁻ vector pACYC184. This construct, pTJ12, was then tested for mobilization by filter mating *E. coli* HB101 (R751)(pTJ12) with *E. coli* DW1030. As expected, pTJ12 was found to be *Mob*⁻, due to the partial deletion of the mobilization gene, *mbpA* (table 7).

Table 7.--Complementation analysis of the pLV22a *oriT* region

Complementing Plasmid	Mobilized Plasmid	Mean frequency of mobilization ^a (± SEM)
NA	pTJ6XB	7.5 (±2.8) X 10 ⁻³
pB22a	pACYC184	<1.0 X 10 ⁻⁵
None	pTJ12	<1.0 X 10 ⁻⁵
pTJ8	pTJ12	<1.0 X 10 ⁻⁵
pB22a	pTJ347	4.1 (±0.5) X 10 ⁻³
pB22a	pTJ437	9.4 (±2.6) X 10 ⁻³

Notes: Plasmids were mobilized from *E. coli* HB101, containing R751 and a complementing plasmid, to *E. coli* DW1030. NA = not applicable.

^a Mobilization frequency is defined as the number of plasmid transconjugants / number of R751 transconjugants in the same mating (N=3). 1X10⁻⁵ defines the limit of detection.

However, when wild-type pLV22a (pTJ8) was provided in *trans* (HB101 (R751)(pTJ8)(pTJ12) X DW1030) pTJ12 remained mobilization-deficient (table 7). Since the mutation of pTJ12 could not be complemented by wild-type pLV22a in

trans, we concluded that the region deleted in pTJ12 contains all or part of the *cis*-acting component required for mobilization, the *oriT*.

Cloning the pLV22a *oriT*

We used the polymerase chain reaction (PCR) to clone the putative *cis*-acting *oriT* of pLV22a. A 347 bp PCR fragment (nt 44 - nt 390) and a 437 bp PCR fragment (nt 44 - nt 480), both containing the putative *oriT* region, were cloned into the *Mob*⁻ vector pACYC184, creating pTJ347 and pTJ437, respectively. pTJ347 and pTJ437 were tested for mobilization when co-resident with R751 and with wild-type pLV22a (pB22a) *in trans*, by mating the following strains with *E. coli* DW1030: *E. coli* HB101 (R751) (pB22a) (pTJ347) and HB101 (R751) (pB22a) (pTJ437). In these matings, both pTJ347 and pTJ437 were found to be fully mobilizable, relative to wild-type pLV22a in pTJ6XB (table 7). To ensure that homologous recombination between the cloned fragments of pTJ347/pTJ437 and the mobilization-proficient pB22a had not occurred, 5 samples of transconjugant plasmid DNA from each of these matings were analyzed by restriction endonuclease digestion. As expected (both HB101 and DW1030 are *recA* mutant strains), no alterations were observed in the digestion patterns of mobilized pTJ347 and pTJ437 (data not shown). These data indicated that pTJ347 and pTJ437 were most likely

mobilized *in trans* when co-resident with wild-type pLV22a (and R751), and therefore contain the pLV22a *oriT*.

CHAPTER 5

SUMMARY & DISCUSSION

The objective of this dissertation project was to isolate and characterize a novel conjugative (i.e., transferable by a conjugation-type mechanism) *B. fragilis* factor. By describing pLV22a and its mobilization region, both here and elsewhere (62), this aim has been achieved. The experiments performed in the project may be divided into 5 principal groups:

- I. The capture of pLV22a by the transfer-deficient plasmid pGAT400•BglIII in an experiment designed to investigate transfer factors present in the *B. fragilis* strain LV22.
- II. The determination that pLV22a possessed a mobilization region which functioned in both *B. fragilis* and *E. coli*.
- III. The localization of the pLV22a mobilization region by the use of Tn1000 insertion mutagenesis, and the subsequent determination of the mobilization region DNA sequence. Computerized analysis of this sequence identified 5 major open reading frames

(ORFs), all of which were potentially required for mobilization in *E. coli*.

IV. The use of complementation analysis to demonstrate that the 3 "top strand" ORFs, designated *mbpA*, *mbpB* and *mbpC*, produce *trans*-acting products which are required for transfer in *E. coli*.

V. The cloning of a *cis*-acting element located within *mbpA* that is required for transfer in *E. coli*, and which probably represents the pLV22a origin of transfer *oriT*.

A discussion of the findings from each group of experiments follows.

Capture of pLV22a by pGAT400·BglIII in *B. thetaiotaomicron*

BT22.47

In a search for novel *Bacteroides* conjugative transfer factors, the *Bacteroides*-*E. coli* *Tra*⁻ shuttle plasmid pGAT400·BglIII captured a 4.2 kb plasmid, pLV22a, in a mating between *B. thetaiotaomicron* BT22.47 (pGAT400·BglIII) and *E. coli* HB101. From this mating, 2 transconjugants were found to contain pGAT400·BglIII::pLV22a fusions, designated pTJ47-1 and pTJ47-3. We hypothesized that pTJ47-1 and pTJ47-3 had been formed in the donor, BT22.47, and that pLV22a conferred transferability to the fusion plasmids, enabling

them to transfer to HB101. The donor, BT22.47, was itself a product of a conjugative mating between a clinical isolate, *B. fragilis* LV22, and a laboratory reference strain, *B. thetaiotaomicron* BT4001. We used high-stringency Southern hybridization analysis to determine that pLV22a was present in BT22.47, and 1 parent, LV22, but not in the other parent, BT4001 (fig. 10). The fact that pLV22a had transferred from LV22 to BT4001, (giving BT22.47), provided additional evidence that the plasmid possessed conjugative properties.

pLV22a was captured by inverse transposition of Tn4400, the resident transposon of pGAT400·BglII. Tn4400 is comprised of a core antibiotic resistance region which is flanked by 2 mobile DNA sequences, IS4400_{left} and IS4400_{right}, in a directly repeated orientation (fig. 1) (81). Restriction-endonuclease mapping and DNA sequencing of the pLV22a-pGAT400·BglII junctions determined that all core Tn4400 DNA internal to IS4400_{left} and IS4400_{right} had been replaced by pLV22a DNA. These data indicated that pLV22a had been acquired by IS4400-driven inverse transposition events, in which the inward-facing IS ends functioned to delete the core DNA and simultaneously acquire pLV22a. While Tn4400 inverse transposition in *E. coli* has previously been reported (81), this is the first known instance of inverse transposition in *Bacteroides*.

In the mating that produced pTJ47-1 and pTJ47-3, 2 other types of pGAT400•BglIII transfer were also noted (figs. 6 and 7): (a) transfers resulting in the deletion of Tn4400 from pGAT400•BglIII, and (b) transfers of unaltered pGAT400•BglIII. Since both pTJ47-1 and pTJ47-3 have proven to be extremely stable in *E. coli*, the possibility exists that pGAT400•BglIII may have in some instances been transferred by a *Bacteroides* factor other than pLV22a hypothetically present in the donor, BT22.47. Type a products could have been produced if the putative BT22.47 transfer factor was integrated into pGAT400•BglIII by Tn4400, where it directed transfer of the cointegrate into *E. coli*, and was then deleted due to an instability of *Bacteroides* DNA in *E. coli*. Such instability of *Bacteroides* transfer factors in *E. coli* has been noted elsewhere (102,105). Alternatively, type b products may have been the result of pGAT400•BglIII transfer *in trans* by a putative BT22.47 transfer factor. (This putative factor may have been the LV22 Trans Factor, the original subject of this study. See Description of the Transfer Mechanisms of the *B. fragilis* Strain LV22, Chapter 4.) The existence of additional transfer factors in BT22.47, however, remains to be determined.

We had thus isolated a 4.2 kb plasmid, pLV22a, from *B. fragilis* LV22 that displayed indirect evidence of conjugative transfer in *Bacteroides*. Our next objective

was to more exactly determine the nature of pLV22a transfer.

Characterization of the Conjugative Transfer Properties of
pLV22a

pLV22a was identified as part of the *Tra*⁺ fusion plasmids pTJ47-1 and pTJ47-3, and we hypothesized that the transferability of the fusions was related to the presence of pLV22a present *in cis*. We found that pLV22a was indeed transferable, both in *Bacteroides* and in *E. coli*. In *Bacteroides*, pLV22a, as part of pTJ10, transferred at a frequency of 7.1×10^{-9} from the laboratory reference strain *B. fragilis* TM4000 (table 4). This finding is near our limit of detection, and is similar to that of pBFTM10 and Tn4399, which both transfer from TM4000 at very low frequency (41,42).

We questioned what effect a Tet Element would have on pLV22a transfer in *B. fragilis*, since Tet Elements increase the transfer frequencies of the *Bacteroides* plasmids pBFTM10 and pB8-51, and the conjugative transposon Tn4399 by 1-2.5 orders of magnitude (42,104). Tet Elements are *Bacteroides*-specific, conjugal (i.e., self-transferable) factors (70- ~200 kb) that exist on the chromosome of their host and express tetracycline, and sometimes also erythromycin, resistances (4,102). We found that pLV22a transfer was increased by 3.5 orders of

magnitude, to 1.5×10^{-5} , from *B. fragilis* TM4.23, a Tet Element⁺ isogeneic strain of TM4000 (table 4).

Notably, *Bacteroides* transfer factors such as pBFTM10 and Tn4399 are conjugatively transferred in *E. coli* when co-resident with the conjugal IncPB plasmid R751 (i.e., they are mobilized) (59,104). pLV22a, as part of the original fusion plasmids pTJ47-1 and pTJ47-3, is also mobilized from *E. coli* when co-resident with R751, at frequencies similar to pBFTM10 and Tn4399 (table 3). To ensure that the parental plasmid of pTJ47-1 and pTJ47-3, pGAT400·BglII, is not required for pLV22a mobilization, pLV22a was cloned into the *Mob*⁻ vector pACYC184, giving pTJ5a and pTJ6. pTJ5a and pTJ6 were mobilized at frequencies equal to or greater than the mobilization frequencies of pTJ47-1 and pTJ47-3, thus demonstrating that pGAT400·BglII is not required for pLV22a mobilization in *E. coli* (table 3). We also found that pTJ6 was mobilized at a higher frequency than were pTJ5a, pTJ47-1 and pTJ47-3 (table 3). The reason for this increase in mobilization frequency is unknown, but may be related to the way in which pTJ5a and pTJ6 were constructed: in pTJ5a, pLV22a is inserted into the chloramphenicol resistance gene of pACYC184, while pTJ6 contains pLV22a in the tetracycline resistance gene of pACYC184 (See Plasmid Constructions, Chapter 3.)

The mechanism by which *Bacteroides* Tet Elements increase the transfer of pLV22a and other co-resident *Bacteroides* transfer factors is unclear, but may be related to the way in which these same plasmids are mobilized in *E. coli* when co-resident with R751. In the family Enterobacteriaceae, of which *E. coli* is a member, 2 major groups of naturally occurring plasmids exist that transfer by conjugative mechanisms. The first group, typified by RP4, R751, and F, is comprised of large, conjugal plasmids (50- >100 kb) that encode virtually all of the many functions needed for their own transfer. Plasmids of the second group, such as RSF1010 (23) and ColE1 (10), are smaller than conjugal plasmids (≤ 10 kb), do not encode all functions required for transfer, and are thus not self-transferable. However, they do encode 2 components that have been found to be essential for conjugative transfer: (a) a *cis*-acting origin of transfer (*oriT*), the site at which one strand of DNA is nicked in a specific manner in preparation for transfer into the recipient cell, (10,24), and (b) a limited number of *trans*-acting proteins that recognize, and then nick, their cognate *oriT* (8,10,49). Because plasmids of the second group contain these 2 elements, they are able to initiate DNA processing at *oriT*, and are conjugatively mobilized to a recipient cell when co-resident with certain conjugal plasmids. We and others infer that the conjugal plasmid

supplies the additional, more generalized transfer functions not encoded by the mobilized plasmid (123).

The 2-step model of Enterobacteriaceae plasmid mobilization may serve to explain the increased transfer of pLV22a, pBFTM10 and Tn4399 that is observed when they are co-resident with a conjugal Tet Element in *B. fragilis*, and also their mobilization in *E. coli*, when co-resident with R751. Both pBFTM10 and Tn4399 resemble mobilizable plasmids from the Enterobacteriaceae family in size (14.6 kb and 9.6 kb, respectively) and in the limited number of transfer functions they encode (i.e., an *oriT* and 2 transfer proteins involved in binding and nicking at their cognate origins of transfer) (41,59,60) (D.W. Hecht, personal communication). pLV22a is also small (4.2 kb), and encodes 3 proteins and an *oriT* required for mobilization. It is thus possible that pLV22a, Tn4399 and pBFTM10 encode only the functions required for nicking 1 strand of DNA at their cognate *oriT*, while the other, more generalized transfer functions required to complete transfer are provided by Tet Elements in *Bacteroides* and by R751 in *E. coli*. It is important to note that while pLV22a, pBFTM10, and Tn4399 all conjugatively transfer in both *E. coli* and *B. fragilis*, only pBFTM10 has been shown to use the same transfer genes in both species (41).

The finding that pLV22a was mobilizable in *E. coli* when co-resident with R751 was fortuitous, since the use

of this species allowed us to apply molecular methods not available in *Bacteroides*. Genetic exchange between *Bacteroides* and *E. coli* may also have a physiologic basis since: (a) *Bacteroides* plasmids can transfer at least as efficiently from *Bacteroides* to *E. coli* as from *Bacteroides* to *Bacteroides* (41). (We regularly use *E. coli* as a recipient in conjugative matings from *Bacteroides*.) (b) Plasmids that can transfer in *E. coli*, such as R751, can also transfer to *Bacteroides* (103). (c) *Bacteroides* and *E. coli* flourish together in their natural habitat, the mammalian gut, and are frequently co-isolated from intra-abdominal abscesses (See Epidemiology, Chapter 2). In spite of these findings, conjugative plasmids alone do not appear to be capable of efficiently transferring genetic information between *Bacteroides* and *Escherichia*, since plasmids from 1 genus are not maintained in the other genus, and most genes from 1 genus are not expressed in the other (35,47,103,105). However, transposable elements such as Tn4400 and Tn4351 can transpose in both *E. coli* and *Bacteroides* (103,105), acquire new DNA by inverse transposition (81, this study), and have promoters that can drive expression of newly-acquired genes in both genera (79). Conjugative plasmids could thus act together with transposable elements to successfully exchange genetic information between *Bacteroides* and *Escherichia*. In this model of inter-genus

genetic exchange, the transposon would acquire, express and mobilize genes within the cell, while the conjugative plasmid would serve as an intercellular carrier of a transposon and its genetic information.

Localization and DNA Sequence Analysis of the pLV22a Mobilization Region

After determining that pLV22a possessed a conjugative mobilization region, we created a library of pLV22a insertion mutants with Tn1000. By screening the library for mutants that were no longer mobilizable in *E. coli*, and then mapping the positions of Tn1000 in those mutants, we identified a 1.5 kb region required for mobilization. We then used these same *Mob*⁻ mutants, together with outward-facing primers to both ends of Tn1000, to determine the nucleic acid sequence of the pLV22a mobilization region (fig. 12).

While mapping mutant positions in pLV22a, a 1.0 kb region of pLV22a was identified that was resistant to Tn1000 insertion (fig. 11). This region is not required for mobilization in *E. coli*, since it is not present in the fully mobilizable plasmid pTJ6XB (table 3); however, the region could be involved in pLV22a transfer in *Bacteroides*. Computerized DNA sequence analysis (DNASIS, Version 3.00 for IBM compatible computers, Hitachi Software Corp.) of the 1.5 kb pLV22a mobilization region

identified 3 ORFs of greater than 10 kDa size in the "top" strand, *orf 1*, *orf 2* and *orf 3*, and 2 major ORFs, *orf 4* and *orf 5*, in the "bottom" strand. Tn1000 insertions into *orf 1*, *orf 2* and *orf 3* resulted in *Mob*⁻ phenotypes, implying that all 3 are required for mobilization in *E. coli* (fig. 13). Due to the nested architecture of *orf 4* and *orf 5* within *orf 2*, no conclusions could be drawn from Tn1000 mutagenesis data concerning the necessity of *orf 4* and *orf 5* for mobilization (fig. 13). *orf 1* and *orf 2* overlap by 4 bp, *orf 2* and *orf 3* overlap by 44 bp, and *orf 5* overlaps *orf 4* by 326 bp (fig. 12 and fig. 13). The derived protein products of *orf 1*, *orf 2*, *orf 3*, *orf 4*, and *orf 5* are 11.7 kDa, 29.4 kDa, 17.6 kDa, 18.9 kDa and 13.4 kDa in size, respectively, based upon the first methionine codon in each ORF (fig. 12). While relatively small, these derived protein sizes are similar to the DNA processing proteins of: Tn4399, MocA (36.4 kDa) and MocB (16.4 kDa); pBFTM10, BtgA (23.2 kDa) and BtgB (33.8 kDa); ColE1, MbeB (17 kDa), MbeC (12 kDa) and MbeD (9 kDa) (10,41,59).

We had now experimentally identified the 1.5 kb region of pLV22a required for mobilization, sequenced this region, and identified 5 major ORF's. Our next experiments were designed to test whether *orf 1*, *orf 2*, and *orf 3* are required for pLV22a mobilization in *E. coli* when co-resident with R751.

Identification and Characterization of the pLV22a Genes
Required for Mobilization in *E. coli*

We used complementation analysis to confirm that *orf* 1, *orf* 2 and *orf* 3 produce *trans*-acting products that are required for mobilization. Because *orf* 4 and *orf* 5 are located entirely within *orf* 2, complementation analysis could not be used to ascertain their role in mobilization (fig 13). We established that mutations in the mobilization region clearly fell into 1 of 3 complementation groups: mutations in *orf* 1; mutations in *orf* 2; and mutations in *orf* 3 (table 6). That each complementation group contains mutations in only 1 major top strand ORF (i.e., *orf* 1, *orf* 2 or *orf* 3), and that no instances of Tn1000-induced polarity on downstream ORF's were seen, strongly argues that each ORF is an independently promoted gene whose *trans*-acting product is required for mobilization. Based on these data, we renamed *orf* 1, *orf* 2 and *orf* 3 *mbpA*, *mbpB*, and *mbpC*, respectively.

Promoter and ribosomal binding site consensus sequences have not as yet been identified in *Bacteroides*. However, since pLV22a is mobilized in *E. coli*, we analyzed *mbpA*, *mbpB*, *mbpC*, *orf* 4, and *orf* 5 for the presence of the major, *E. coli* σ^{70} promoter, and for Shine-Dalgarno ribosomal binding sites. No strong homology to either the -10/-35 promoter or Shine-Dalgarno consensus sequences was

observed. If the expression of *mbpA*, *mbpB*, and *mbpC* is necessary for transfer in *Bacteroides* as well as in *E. coli*, their promoters and ribosomal binding sites may contain customized sequences that are recognized in both *E. coli* and in *Bacteroides*; this could account for our inability to identify *E. coli* transcription/translation signals in *mbpA*, *mbpB*, and *mbpC*, as well as in *orf 4* and *orf 5*.

DNA sequence analysis detected 2 additional characteristics of pTJ47-1 and pTJ47-3, the original pLV22a fusion plasmids, that were a result of pGAT400·BglIII insertion into pLV22a at 2 different sites. In pTJ47-1, the *mbpA* coding region begins only 19 bp distal from IS4400_{left} of pGAT400·BglIII (figs. 12, 13). Thus, the *mbpA* promoter would appear to be interrupted in this clone. We speculate that *mbpA* may be promoted by an outward facing promoter potentially present in IS4400_{left}. (IS4400 is not known to have such promoters. However, outward facing promoters active in *E. coli* have been identified in a closely related insertion element, IS4351 (79).) In pTJ47-3, *mbpA* contains its native upstream sequence, and is thus probably promoted by its own promoter. The fact that no significant difference was noted in the mobilization frequencies of pTJ47-1 and pTJ47-3 (table 3) could indicate that the *mbpA* native promoter is of similar strength to the putative IS4400

promoter, that MbpA is not a limiting factor in plasmid mobilization efficiency, or both.

Sequence analysis also discovered that pTJ47-3 contains an interruption of *mbpC* 7 nucleotides upstream of its translational stop codon (fig. 12). The amino acids encoded by these codons do not however appear to be necessary for mobilization, since pTJ47-3 is fully mobilizable respective to pTJ47-1, in which *mbpC* is intact.

Several lines of evidence suggest that MbpB is the DNA relaxing enzyme, or relaxase, of pLV22a. (Relaxases are those enzymes that specifically nick 1 strand of DNA at a cognate *oriT* sequence in preparation for conjugative transfer. For more on relaxases, see Horizontal Gene Transfer Factors, Chapter 2.) The BestFit program of the Genetics Computer Group package (Version 8.0) was used to compare MbpB with the transfer proteins of pBFTM10 and Tn4399. BestFit, which makes an optimal alignment of the best segment of similarity between two sequences, identified a 73 aa segment of Moca, a proposed relaxase of the *B. fragilis* conjugative transposon Tn4399, that is 38% identical to a region of MbpB (fig. 14) (59). We also identified a 6/10 match (8/10 when conservative substitutions are included) between MbpB and a consensus sequence that was derived from confirmed and putative bacterial relaxases (68) (fig. 14). Both MbpB and Moca

A.

MbpB	(pLV22a)	105	R H S G T	<u>E</u> <u>S</u> <u>K</u> <u>K</u> <u>E</u> <u>Q</u> <u>A</u> <u>H</u> <u>L</u> <u>H</u> <u>I</u> <u>L</u> <u>A</u> <u>N</u>	R V S F
MocA	(Tn4399)	97	Y I V A R	H T D R E H P H C H I V F N	R V D N
MobA	(pC221)	94	V A V Y T	H T D K D H Y H N H I V I N	S V D L
TraI	(RP4)	104	R V S A V	H N D T D N L H I H I A I N	K I H P
TraI	(R751)	104	R I S A V	H N D T D N L H I H I A I N	K I H P
VirD2	(pTIA6)	126	Y L T A Y	H V D R D H P H L H V V V N	R R E L
VirD2	(pRIA4)	126	Y L T A F	H I D R D H P H L H V V V N	R R E L
NikB	(R64)	153	Y V S A V	H T D T D N L H V H V A V N	R V H P
Consensus:				H D D N H H c c c N	
				H	

B.

MbpB	59	V k n n c L r F e v S P s i e E s a t f t D A d w a e l g
MocA	57	V g h t s L n F - - S P e d g E r l k t d D A l m l q i a
MbpB	88	n D f M q r M G l a N h Q Y I i i R
MocA	84	h D y M a k M G i e N t Q Y I v a R
		<u>H</u> <u>s</u> <u>q</u> <u>t</u> <u>e</u> <u>s</u> <u>k</u> <u>k</u> <u>E</u> <u>q</u> <u>a</u>
		<u>H</u> <u>t</u> <u>d</u> - - - - r e h p
MbpB	117	<u>H</u> <u>l</u> <u>H</u> <u>I</u> <u>l</u> <u>a</u> <u>N</u> R V s f p G n c t g t t m D
MocA	109	<u>H</u> <u>c</u> <u>H</u> <u>I</u> <u>v</u> <u>f</u> <u>N</u> R V d n d G k t i s d k n D

Fig. 14. Boxed sequences denotes conserved relaxase regions according to Pansegrau and Lanka (see text for details). The number to the left of each sequence denotes the position of the first illustrated amino acid. **A.** Alignment of putative relaxase regions of MbpB (pLV22a) with other putative and known relaxase sequences. The letter c in the consensus sequence denotes neutral, hydrophobic, amino acids. Underlined letters in the MbpB sequence denote amino acids identical to the consensus sequence, or amino acids which represent conserved substitutions. **B.** Alignment of amino acid sequences from MbpB and MocA (Tn4399), overlapping the putative relaxase region as illustrated in Part A. Uppercase letters denote identical amino acid residues, while lowercase letters denote different residues.

carry the relaxase consensus sequence within their regions that also display 38% identity (fig. 14). While imperfect, we consider the match between MbpB and the consensus sequence to be significant, since Bacteroidaceae are distantly related to the bacteria from which the consensus is derived (122). Notably, MbpB contains conserved histidine residues in the consensus sequence at positions 117 and 119. These histidine residues are also present in the consensus sequence of a known relaxase, TraI of RP4 (aa 116 and 118), and are critical for nicking at *oriT_{RP4}* (fig. 14) (70). The homology between MocA and MbpB around the relaxase consensus sequence suggests that these proteins, from otherwise very different transfer factors, may share a common ancestral gene. In contrast, BestFit did not identify significant homology between MbpB and the transfer proteins of the *B. fragilis* plasmid pBFTM10, BtgA and BtgB, making a common predecessor for these proteins less likely.

We also used FastA and TFastA of the Genetics Computer Group package to compare *mbpA*, *mbpB*, *mbpC*, *orf 4* and *orf 5* to sequences in the GenBank/EMBL database. Both FastA and TFastA perform Pearson and Lipman searches for similarity between a query sequence and a group of database sequences. FastA analyzes both strands of a DNA sequence, while TFastA analyzes all 6 open reading frames

derived from a DNA sequence. No significant homologies between pLV22a and databank sequences were identified. Studies are currently under way to investigate whether MbpB is indeed a relaxase that is active in nicking at *oriT*_{pLV22a}, and to discover what roles MbpA and MbpC may play in the process.

Cloning a cis-acting Region Required for Mobilization

Complementation analysis of *mbpA* determined that Tn1000 insertion mutation 67, which is located within *mbpA* at nt 317, was only partially complemented when MbpA was provided *in trans* (see table 6, Mobilization genotype *mbpA*, mobilization of pAC67 by complementing plasmids pBR39 and pBR18). This suggested to us that the relatively large transposon insertion of mutation 67 was disrupting both *mbpA* and the *cis*-acting *oriT*_{pLV22a}. Using computer analysis, we also identified 2 imperfect inverted repeats within *mbpA*, the first spanning from nt 234 - nt 260, the other from nt 267 - nt 292 (fig. 12). The finding of inverted repeats near mutation 67 supported the idea that the pLV22a *oriT* is located within *mbpA*, since inverted repeats have been previously identified in other bacterial origins of transfer (41,60,126). To test this hypothesis, we PCR-amplified a 347 bp region (nt 44 to nt 390) around mutation 67, and cloned the fragment into the *Mob*⁻ vector pACYC184 (fig. 12). We found that this

construct, pTJ347, was fully mobilizable from *E. coli* when co-resident with R751 and wild-type pLV22a on a compatible plasmid (table 7). This result indicated that the 347 bp PCR fragment, from within *mbpA*, contains the pLV22a *oriT*. DNA sequence analysis of the fragment identified a region that displays 66.7% homology to the 12 bp *oriT*/nick-site consensus sequence of Pansegrau and Lanka (68):

pLV22a	nt 158	<u>A</u> <u>C</u> <u>C</u> G <u>A</u> A <u>C</u> T <u>T</u> <u>G</u> A <u>A</u>
Consensus:		<u>A</u> <u>C</u> <u>C</u> T <u>A</u> T <u>C</u> C <u>T</u> <u>G</u> C <u>A</u>
		G T A C T C
		G T

Bacteroidaceae are distantly related to the bacteria from which this consensus is derived (122), and we therefore consider the match, while imperfect, to nevertheless be significant. We have thus localized the pLV22a *oriT* to a 347 bp region that is located primarily within *mbpA*. This is not unique: the Tn4399 *oriT* also resides in a mobilization gene, *mocB* (59). Experiments to identify the nick site within the 347 bp PCR fragment, using PCR cycle-sequencing, are currently under way.

Potential Functions of the pLV22a Mobilization Proteins

While the genes required for pLV22a mobilization in *E. coli* are known, the functions of their protein products, MbpA, MbpB and MbpC, are not. We propose that MbpA, MbpB and MbpC function to recognize and bind at

oriT_{pLV22a}, and to then specifically nick 1 strand of DNA in preparation for transfer. This model, which is based upon the relatively well characterized DNA processing systems of conjugative plasmids found in bacteria of the family Enterobacteriaceae, may also apply to *Bacteroides* transfer factors other than pLV22a. (For a review of *Bacteroides* factors, see Chapter 2, Horizontal Transfer Factors.)

Among the Enterobacteriaceae, 3 plasmids whose DNA processing mechanisms have been extensively reported on are ColE1, RSF1010, and RP4.

The 6.6 kb plasmid ColE1 encodes 4 proteins, MbeA (60 kDa), MbeB (17 kDa), MbeC (12 kDa) and MbeD (9 kDa), which are required for mobilization when co-resident with IncF, IncI, IncP or IncW conjugal plasmids (10). Helinski et al. have determined that three mobilization proteins (probably MbeA, MbeB and MbeC), form a relaxosome at the ColE1 *oriT*. A 60 kDa relaxosome protein that is likely MbeA becomes covalently linked to the 5' end of the nicked DNA strand, and may act to chaperon this strand into the recipient while protecting it from 5' exonucleases. The 60 kDa protein may also re-circularize the strand in the recipient (8,36,49).

RSF1010 is an 8.9 kb IncQ plasmid that is mobilized when co-resident with conjugal plasmids of the IncI α , IncM, IncP and IncX groups. RSF1010 encodes 3 relaxosome proteins, MobA, MobB and MobC, that are required for

mobilization *in vivo* (23). MobA is a large (78 kDa), multi-functional protein that recognizes a specific sequence in *oriT*_{RSF1010}, binds to the sequence, and then nicks 1 strand of DNA within the *oriT*. After nicking, MobA covalently bonds to the 5' end of the broken DNA strand, where it may act to recircularize the strand following entry of the nucleoprotein complex into the recipient (94,95). (MobA is unusual in that it is also known as RepB, and functions as a primase in the vegetative replication of RSF1010 (96).) The second relaxosome protein, MobB, increases the efficiency of *in vitro* nicking at *oriT* by either stabilizing the relaxosome complex, or by altering the local DNA topology (95). The third protein, MobC, is a helicase that is brought into the relaxosome by MobA, and may serve to melt the double-stranded DNA molecule prior to nicking (95).

While all the details of DNA processing in ColE1 and RSF1010 have not been elucidated, it is clear that each encodes several proteins that function in the context of a relaxosome to specifically nick 1 strand of DNA at a site within their cognate *oriT*. Perhaps the most well studied DNA processing system is that of the IncP plasmid RP4. RP4 is very different from ColE1 and RSF1010, and also pLV22a, in that it is much larger (60 kb), and is fully self-transferable. However, one way in which RP4 resembles ColE1 and RSF1010 is that it too uses a

relaxosome to specifically nick 1 strand of DNA at its *oriT* prior to transfer. In RP4 relaxosome formation, TraJ first recognizes, and then binds, to the right half of a 19 bp inverted repeat sequence within the *oriT* (126). TraI then recognizes a 6 bp sequence between the nick site of *oriT* and the TraJ-binding site, and joins the TraJ-*oriT* nucleoprotein complex (69). TraH specifically interacts with both TraI and TraJ, and is proposed to stabilize the relaxosome (67), while TraK acts to enhance nicking at *oriT* by locally altering the DNA superhelicity (29,127). Once the relaxosome is formed, TraI cleaves a defined strand of DNA at the nick site, after which TraI becomes covalently attached to the 5' phosphoryl end (69).

MbpA, MbpB and MbpC may also function in a relaxosome to nick 1 strand of DNA within *oriT*_{pLV22a}. As discussed above, MbpB appears to be the relaxase of pLV22a. As the relaxase, MbpB may both recognize and bind to a site within *oriT*_{pLV22a}, and then nick 1 strand of DNA. This is similar to MobA of RSF1010, the relaxase which both recognizes and binds to *oriT*_{RSF1010}, and then provides the nicking function. Alternatively, MbpB may require MbpA and/or MbpC to first recognize and bind *oriT*_{pLV22a} before it can enter the relaxosome/DNA complex (as is seen with TraJ/TraI of RP4) (fig. 15).

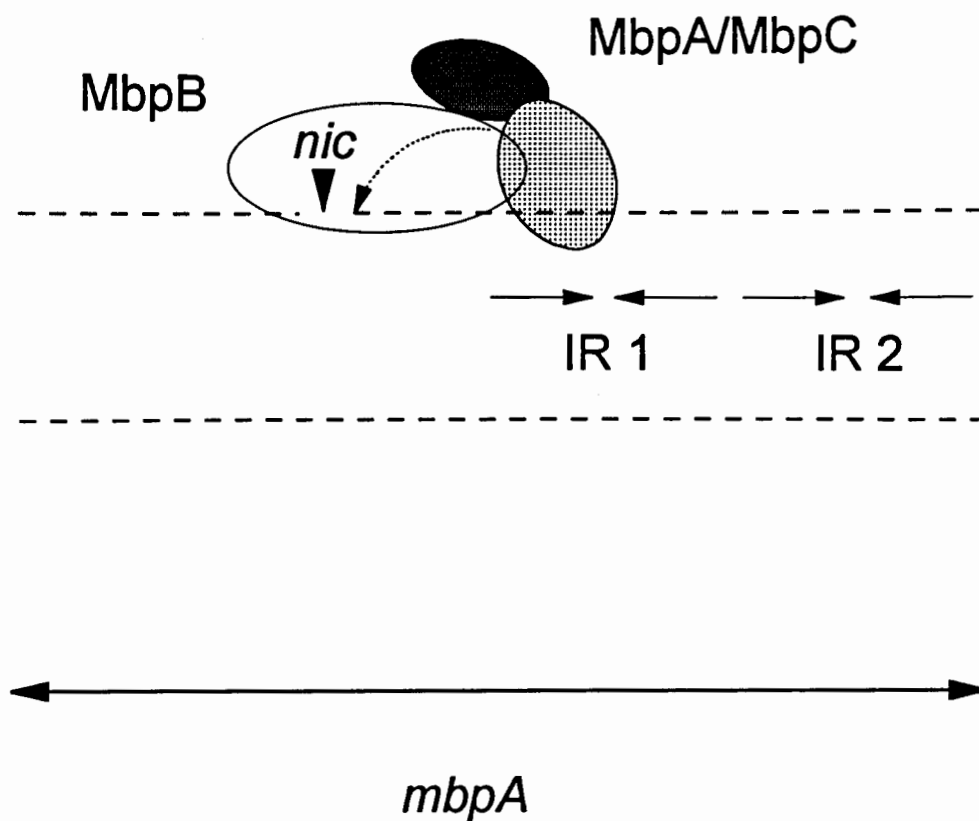


Fig. 15. Model of potential nucleoprotein interactions at the *oriT* of pLV22a. IR 1 and IR 2 represent inverted repeats 1 and 2, as found in figure 12. Dotted arrow represents potential covalent bonding of an Mbp protein to the nicked strand of DNA at its 5' end. Dashed horizontal lines represent double-stranded DNA. The position of *nic* in this figure is hypothetical.

MbpA, MbpB and MbpC may also:

- (a) possess helicase activity, which would serve to melt the DNA prior to nicking, as does MobC in the RSF1010 system;
- (b) act to stabilize the relaxosome complex, as is seen with TraH of RP4;
- (c) alter the local DNA topography to facilitate *oriT* recognition and binding by the relaxosome, as does TraK of RP4;
- (d) covalently bond to the nicked strand of pLV22a and then act to chaperon the strand into the recipient. Once in the recipient, the bound protein may act to protect the strand from nuclease attack, and participate in re-circularizing the DNA strand prior to second-strand synthesis. (Some of these functions are thought to be fulfilled by MobA of RSF1010 and MbeA of ColE1.) As chaperon, the bound pLV22a protein may also interact with transfer proteins from the conjugal plasmid that assist in transfer and re-circularization of the nicked strand, and with bacterial proteins to assist in second strand synthesis.

Experiments are currently being conducted in our laboratory to test this model.

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VITA

The author, Thomas J. Novicki, was born on January 28th, 1958, in Evergreen Park, Illinois. He attended Loyola University Chicago, and received his bachelor degree in Biology in January of 1982. The author successfully passed the ASCP Medical Technology Board Examination in 1983, after completing a one year course of study in Medical Technology at Christ Hospital (Oak Lawn, Illinois). He then practiced Medical Technology from 1983 to 1989, at both Good Samaritan Hospital (Downers Grove, Illinois) and the Loyola University Medical Center. The author was accepted into the graduate program at Loyola University Chicago, and began his studies in the Molecular Biology Program in the fall of 1989. The author became interested in microbial genetics while studying under Dr. Robert V. Miller (formerly of Loyola University of Chicago). He then entered the laboratory of Dr. David W. Hecht, and began his dissertation research on the mechanisms of horizontal gene transfer by conjugation-type mechanisms in the pathogenic bacterium *Bacteroides fragilis*.

The author was a recipient of the Arthur J. Schmitt Dissertation Fellowship, and is a member of the Anaerobic Society of the Americas and the American Society for Microbiology. He is married, has two children, and will begin a two year Fellowship in Clinical Microbiology at the University of Utah (Salt Lake City) in September of 1996.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Thomas J. Novicki
has been read and approved by the following committee:

David W. Hecht M.D. Director
Associate Professor, Department of Medicine and Microbiology
Loyola University of Chicago

Leonard C. Erickson Ph.D.
Professor, Department of Medicine and Pharmacology
Director of the Molecular Biology Program
Loyola University of Chicago

John M. Lopes Ph.D.
Assistant Professor, Department of Molecular and Cellular
Biology
Loyola University of Chicago

J. Paul O'Keefe M.D.
Professor, Department of Medicine
Director of the Division of Infectious Diseases
Loyola University of Chicago

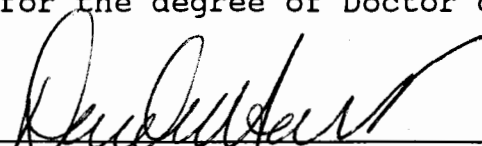
Alan J. Wolfe Ph.D.
Assistant Professor, Department of Microbiology and
Immunology
Loyola University of Chicago

The final copies have been examined by the director of the
dissertation and the signature which appears below verifies
the fact that any necessary changes have been incorporated
and that the dissertation is now given final approval by the
committee with reference to content and form.

The dissertation is, therefore, accepted in partial
fulfillment of the requirements for the degree of Doctor of
Philosophy.

7/22/96

Date



Director's Signature